INVESTIGATIONS OF THE FORGOTTEN *GEUM*: GENETIC DIVERSITY AND POPULATION BIOLOGY OF *GEUM GENICULATUM* MICHAUX, BENT AVENS.

A Thesis
by
MARIETTA DAY SHATTELROE

Submitted to the School of Graduate Studies
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

December 2019
Department of Biology

INVESTIGATIONS OF THE FORGOTTEN *GEUM*: GENETIC DIVERSITY AND POPULATION BIOLOGY OF *GEUM GENICULATUM* MICHAUX, BENT AVENS.

A Thesis
by
MARIETTA DAY SHATTELROE
December 2019

APPROVED BY:

_____
Matt C. Estep
Chairperson, Thesis Committee

_____
Jennifer Rhode Ward
Member, Thesis Committee

_____
Ray Williams
Member, Thesis Committee

_____
Zack Murrell
Chairperson, Department of Biology

_____
Mike McKenzie, Ph.D.
Dean, Cratis D. Williams School of Graduate Studies

Abstract

INVESTIGATIONS OF THE FORGOTTEN *GEUM*: GENETIC DIVERSITY AND POPULATION BIOLOGY OF *GEUM GENICULATUM* MICHAUX, BENT AVENS.

Marietta Day Shattelroe
A.S. Asheville-Buncombe Technical Community College
B.S. University of North Carolina Asheville

Chairperson: Matt C. Estep

Geum geniculatum Michx. (Roseaceae), bent avens, is a perennial herb restricted to the high elevations (>1200 m) of three mountaintops between North Carolina and Tennessee (Weakley, 2015). Because of its limited geographic distribution and affinity for high elevations, it is thought to belong to a group of plants endemic to the southern Appalachians that are considered post-Pleistocene relicts along with other rare species: *Geum radiatum* (spreading avens), *Liatris helleri* (Heller's blazing star), *Solidago spithamea* (Blue Ridge goldenrod) and *Calamagrostis cainii* (Cain's reedgrass; Wiser, 1994). It is an erect plant growing to almost 1 meter in height, with a basal rosette of trifoliate, pinnately compound, or simple leaves and the cauline leaves on the flowering stems have varying leaf shape from simple to trifoliate (Oakley, 1991). It is characterized by a green to dark purple campanulate or bell-shaped calyx and flowers that nod at anthesis.

Although geographically restricted, occurrences on these mountaintops can have up to hundreds and occasionally thousands of individuals (NCNHP, 2018). While population size has been monitored, scientific studies are lacking for *G. geniculatum*. Therefore, there is

a need for research to understand basic life history traits, pollination biology, population demography, and genetic diversity to inform conservation strategies for the species.

In order to address the need to understand life history traits and population viability outlined by Oakley (1991), 13 sub-populations were censused, and a long-term demography study was established at one of the populations. To begin to understand pollination and reproductive biology of the species. an insect visitor survey via time-lapse camera trapping study was performed. Lastly, to understand genetic variation and connectivity within and among populations a population genetics study was performed. The study utilized 14 microsatellite loci for *Geum urbanum* and *Geum reptans* across 89 individuals and the range of the species.

Results of censusing in 2018 suggest the most robust populations occur along stream banks with 90 to 95% canopy cover however the plant can also withstand varying habitat including grassy balds with little to no canopy cover and along heavily trafficked trails. Overall, population sizes are smaller than previously reported but comparisons should be made with caution as census techniques may have varied in the past. The first-year demography data, while only established for one population, will provide a baseline to understand life history traits for the species and to understand the population viability of the smallest metapopulation of *Geum geniculatum.* The genetic results suggest the species has high genetic diversity and is comprised of three highly structured metapopulations with moderate differentiation between them. These data can be utilized by land managers and future researchers to conserve and manage the species as well as to guide future research questions.

Acknowledgments

The author would like to acknowledge Drs. Matt Estep, Jennifer Rhode Ward and Ray Williams for their continued support as committee members and mentors. Sue Fruchey from the US Forest Service and Dr. Chris Ulrey for guidance and help in the field. Page Mangum, Alyssa Phillips and Logan Clark for their assistance and support in the field and in the lab. This project would not be possible if not for funding from the Shinn Scholarship North Carolina Native Plant Society, Appalachian State University Office of Student Research, and Appalachian State University Graduate Student Government Association.

Table of Contents

Foreword

Chapter 1, Chapter 2, Chapter 3, and Chapter 5 are formatted according to APA guidelines.

Chapter 4 has been formatted for publication in *Castanea*, a peer reviewed journal published by the Southern Appalachian Botanical Society.

Chapter 1: Background and Introduction

The genus *Geum* is in the Roseaceae, subfamily Rosoideae (Weakley, 2015). The genus is comprised of 40 to 60 species occurring across the northern and southern hemispheres typically inhabiting mesic habitats (Gajewski, 1957; Weakley, 2015). There are 14 species of *Geum* documented in the southeastern United States; 2 of these species: *Geum geniculatum* and *Geum radiatum*, are rare (Weakley, 2015). All species of *Geum* share a number of morphological traits including a corymbose inflorescence, pinnately compound basal leaves while the cauline leaves on the flowering stems are simple or trifoliate (Gajewski, 1959; Gleason & Cronquist, 1991).

Geum geniculatum Michaux, bent avens, is a rare, perennial herb endemic to high elevations (>1200 m) in the southern Appalachians (Figure 1; Weakley, 2015). Although geographically restricted, subpopulations on the mountains it occurs have been reported to have hundreds to thousands of individuals (NCNHP, 2018). It is an erect plant growing to almost a meter in height, with a basal rosette of varying leaf shape: trifoliate, pinnately compound, or simple. The cauline leaves on the flowering stems may be trifoliate to simple (Oakley, 1991). It is characterized by a green to dark purple campanulate or bell-shaped calyx and flowers that nod at anthesis. The flowers are white and can be distinguished from related species that share the same range, *Geum canadense* and *G. virginianum,* by the varying colored, non-reflexed calyx, and also by the nodding flowers at anthesis (Oakley, 1991; Weakley, 2015). Its sister taxa is *G. rivale* in which it shares similar morphology: styles longer than petals, nodding flowers at anthesis, green to purple calyx (Weakley, 2015). *Geum rivale* is found in calcareous fens, wet meadows and swamps (Gleason & Cronquist, 1991). It is a circumboreal species with distribution across Europe and North America (Taylor, 1997; Weakley, 2015). The two species

do not co-occur but have likely experienced a disjunct distribution post-Pleistocene (Weakley 2015).

Figure 1. Photograph of *Geum geniculatum* in bloom occurring on a high elevation grassy bald at Population 3 in June 2019.

Geum geniculatum is restricted to 5 counties on 3 mountains between North Carolina and Tennessee; because of its rare status population locations throughout this document are masked for protection (Figure 2; NatureServe, 2018; Robinson & Finnegan, 2016). As the crow flies Population 1 and Population 2 are separated by ~23 km, Population 2 and Population 3 by ~27 km and Population 1 and Population 3 by ~42 km (Google Earth, 2019). Its heterogeneous distribution within this area is due to historic climatic changes of warming and drying periods during the Pleistocene, where moist high elevation regions acted as refuge for the species (Weakley, 2015). In addition, habitat fragmentation due to land use changes may have also assisted in this distribution by extirpating historical populations that connected the three sites.

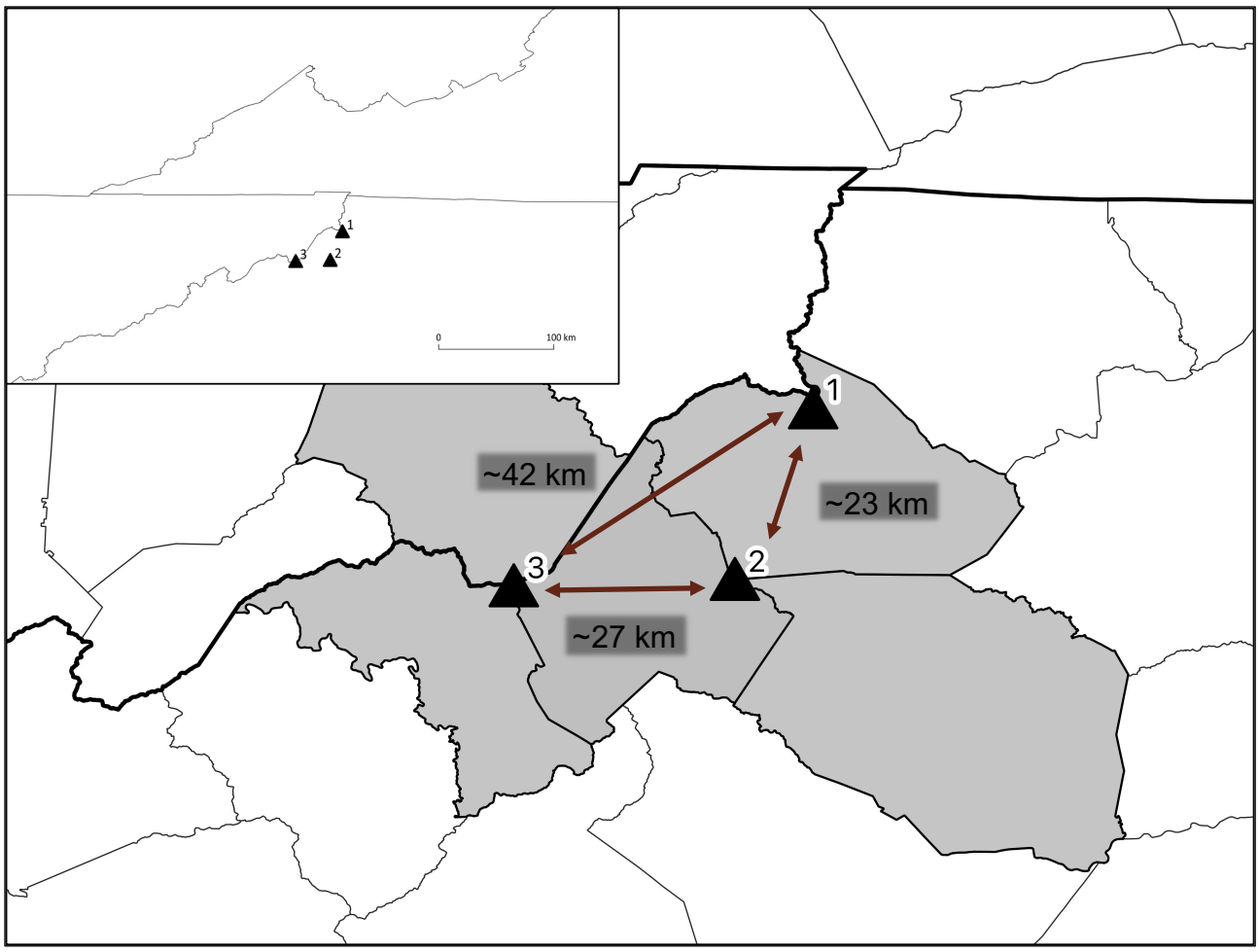

Figure 2. Map showing locations of populations of *Geum geniculatum* in North Carolina and Tennessee.

Other relict species that share this natural history and range include *Geum radiatum*, *Liatris helleri* (Heller's blazing star)*, Solidago spithamea* (Blue Ridge goldenrod) and *Calamagrostis cainii* (Cain's reedgrass; Wiser, 1994). While life history and breeding systems create variable genetic diversity, these relict species are expected to have lower heterozygosity and greater differentiation due to reduction in gene flow because of isolated populations, narrow distribution and typically small population sizes. (Hamrick & Godt, 1996). This expectation and relict hypothesis has been supported through population genetic studies using allozymes in high elevation plant species of *Geum radiatum*, *Carex misera, Trichophorum cespitosum,* and *C. cainii* (Godt, Johnson, & Hamrick, 1996). In the southeastern United States, two *Geum* species

are thought to be Pleistocene relict species: *G. radiatum* and *G. geniculaum.* Other known relict species in the genus include *G. peckii* occupying the White Mountains of New Hampshire; this species is a sister taxa to *G. radiatum* and *G. bulgaricum* in the Balkan Peninsula (Petrova, Petrović, Soljan, & Stevanović, 2011; Weakley, 2015).

The distance and extreme topography between these high elevation mountain sites allow them to act as islands with little to no connecting suitable habitat between them and therefore act as a barrier against gene flow for pollinators and seed dispersal. There is only one documentation of observation of bees visiting the plant, but further research is needed to identify pollinators (Oakley, 1991). *Geum geniculatum* resides in a wide variety of habitats: grassy balds, streambanks and seepy boulderfield forests and has been observed along the sides of trail spanning multiple habitats (Oakley, 1991; Weakley, 2015). The most robust populations are found in those seepy boulderfield forests and along stream banks under a thick forest canopy. Grassy bald population sizes are much smaller (pers. obs.).

Cytogenetic studies have shown that the majority of species within the genus *Geum* are hexaploid (2n = 6x = 42). (Gajewski, 1957; Raynor, 1952). Two species are tetraploids (2n = 4x = 28), and four species are dodecaploid (2n = 12x = 84: (Gajewski, 1959). Polyploidy within the genus likely arose through ancient allopolyploid events (Gajewski, 1957; Smedmark, Eriksson, Evans, & Campbell, 2003). The first allopolyploid event was a hybridization between two diploid species of *Coluria* lineage and *Waldsteinia* lineage gave rise to an allotetrapoid species. The resulting species then backcrossed to form the hexaploid lineages that make up the majority of the genus (Smedmark et al., 2003).

Many taxonomic relationships between species of *Geum* remain unresolved however *G. geniculatum* belong to the subgenus *Eugeum* which also includes *G. canadense, G. virginianum,*

G. rivale, and *G. urbanum* (Gajewski, 1957). *Geum radiatum,* another post-Pleistocene relict species, shares the same range with *G. geniculatum* and is placed within subgenus *Acomastylis* (Gajewski, 1957). Phylogenetic analysis has also supported that *G. geniculatum* is most closely related to *G. rivale* and *G. urbanum* (Smedmark et al., 2003).

Gene flow between populations is crucial for long-term viability and genetic structure of plant populations because it reduces the effects of genetic drift and increases effective population size (Ellstrand, 1992). Knowledge of breeding systems and pollination biology for plant species can help to understand what is causing genetic diversity and structuring of plant populations. This information can also be used to inform conservation strategies for at risk populations. The breeding system for most species within the genus was thought to be outcrossing with self-compatibility (Gajewski, 1959). However, varying levels of self-incompatibility have been demonstrated in some speices. For instance, *Geum rivale*'s dominant breeding system is outcrossing and has been shown to have low levels of self-compatibility (Ruhsam, Hollingsworth, Squirrell, & Ennos, 2010). *Geum reptans* reproduces sexually but produces non-viable seeds when selfed and readily reproduces clonally (Weppler & Stöcklin, 2005). *Geum urbanum* is highly self-compatible, displays low levels of outcrossing and in fragmented populations has been shown to have high rates of self-pollination (Ruhsam et al., 2010; Vandepitte, Honnay, Jacquemyn, & Roldán-Ruiz, 2010). *Geum radiatum* reproduces clonally and produces seeds that appear wind-dispersed; however, the level of self-compatibility is unknown (Ulrey, Quintana-Ascencio, Kauffman, Smith, & Menges, 2016). Seeds of plants belonging to the subgenus *Eugeum* are animal dispersed via adhesion; while, other species of *Geum* occurring in the Arctic and Montane regions are typically wind-dispersed (Sorenson, 1986). The functional groups of insect visitors and possible pollinators also vary throughout the

genus but include bumblebees, syrphid flies, and muscid flies (Ruhsam et al., 2010; Taylor, 1997; Yumoto, 1986). The breeding system and pollinator for *G. geniculatum* is unknown and the geniculate, persistent style that characterizes the fruit along with membership within *Eugeum* suggests that seeds are animal dispersed.

Both *G. geniculatum* and *G. radiatum* share an affinity for high elevations between North Carolina and Tennessee, yet their habitats and morphologies are quite different. *Geum radiatum* prefers sunny, open rock outcrops above 1300 m in elevation and has been federally listed as endangered since 1990 (NatureServe, 2018; Ulrey et al., 2016). Due to its protected status, extensive monitoring has been conducted on it and numerous historic augmentations have been done (Ulrey et al., 2016). Two population genetics studies have also been conducted on *G. radiatum*. An allozyme study found low genetic diversity (H_s = 0.119 among populations) and low to moderate differentiation among populations (G_{st} = 0.191, Godt et al., 1996). More recently, a study using 8 microsatellite loci across the extent of the range (14 populations) was completed (Hay et al. 2019). This analysis concluded high allelic diversity across the species (141 alleles across all loci, with a mean allelic richness of 6.375) and high genetic diversity (mean Ho = 0.635). This high genetic diversity was partly explained by its hexaploid nature. Polyploids, such as species in *Geum*, are likely to have a higher number of alleles than diploids, allowing for higher numbers of possible genotypes within the same locus (De Silva, Hall, Rikkerink, McNeilage, & Fraser, 2005). Thus, they typically have increased heterozygosity compared to diploid species (Soltis & Soltis, 2000). The populations displayed low to moderate differentiation between populations (F_{st} = 0.022 – 0.126), and a STRUCTURE analysis suggested 4 distinct groups throughout its geographic range (Hay et al., 2019).

Even though the range of *G. geniculatum* is more restricted than that of *G. radiatum*, it does not have any protection status, though it is a federal species of concern. *Geum geniculatum* is globally ranked as imperiled (G2) or at high risk for extinction due to its limited range. In Tennessee, it is a state ranked critically imperiled species (S1) and listed as endangered. In North Carolina it is state ranked critically imperiled/imperiled (S1S2) and state listed as a species of concern-vulnerable (SC-V; NatureServe, 2018; Robinson & Finnegan, 2016). There has been little formal biological research conducted on *Geum geniculatum.* In 1980, Massey and Whitson performed a survey of the species which also included 11 other rare taxa. In 1991, an Element Stewardship Abstract was outlined for the species by Shawn Oakley (1991) that laid out research needs including pollination biology, population demography, and genetic diversity. In an effort to address these needs, 1) a census of North Carolina Element Occurrences (EO) was conducted and a long-term demographic plot was established for the species (Chapter 2); 2) a time-lapse visitor survey was conducted to identify insect visitors (Chapter 3) and 3) a population genetic study was conducted using microsatellite markers to understand gene diversity, population structure and connectivity between populations (Chapter 4).

Literature Cited

De Silva, H. N., Hall, A. J., Rikkerink, E., McNeilage, M. A., & Fraser, L. G. (2005). Estimation of allele frequencies in polyploids under certain patterns of inheritance. *Heredity (Edinb), 95*(4), 327-334. doi:10.1038/sj.hdy.6800728

Gajewski, W. (1957). A cytogenetic study on the genus *Geum* L. *Monographiae Botanicae, 4*, 1-416. doi:https://doi.org/10.5586/mb.1957.001

Gajewski, W. (1959). Evolution in the genus *Geum*. *Evolution, 13*(3), 378-388. doi:10.2307/2406114

Gleason, H. A., & Cronquist, A. (1991). *Manual of the Vascular Plants of Northeastern United States and Adjacent Canada* (2nd edition ed.). Bronx, New York: The New York Botanical Garden.

Godt, M. J. W., Johnson, B. R., & Hamrick, J. L. (1996). Genetic diversity and population size in four rare southern Appalachian plant species. *Conservation Biology, 10*(3), 796-805.

Hamrick, J. L., & Godt, M. J. (1996). Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions: Biological Sciences, 351*(1345), 1291-1298.

Hay N.M., Ulrey C., Kauffman G., Murrell Z.E., and Estep M.C. (2019) The genetic diversity and population structure of *Geum radiatum*: effects of past augmentation of an endangered hexaploid. (in press @ Castanea).

NatureServe. (2018). NatureServe Explorer: An online encyclopedia of life [web application]. Version 7.0. Retrieved from http://explorer.natureserve.org

NCNHP. (2018). North Carolina Natural Heritage Program Element Occurences. *Natural Heritage Program.* Retrieved from https://www.ncnhp.org/activities/conservation/natural-heritage-element-occurrences

Oakley, S. (1991). *Element Stewardship Abstract for Geum geniculatum*. The Nature Conservancy. Arlington, VA.

Petrova, A., Petrović, D., Soljan, D., & Stevanović, V. (2011). *Geum bulgaricum*. The IUCN Red List of Threatened Species 2011: e.T165125A5977317. In: International Union for Conservation of Nature and Natural Resources.

Raynor, L. A. (1952). Cytotaxonomic Studies of *Geum*. *American Journal of Botany, 39*(10), 713-719.

Robinson, L. G., & Finnegan, J. T. (2016). *Natural Heritage Program List of Rare Species of North Carolina*. Raleigh NC: North Carolina Natural Heritage Program.

Ruhsam, M., Hollingsworth, P. M., Squirrell, J., & Ennos, R. A. (2010). Significant differences in outcrossing rate, self-incompatibility, and inbreeding depression between two widely hybridizing species of *Geum*. *Biological Journal of the Linnean Society, 101*(4), 977-990.

Smedmark, J. E., Eriksson, T., Evans, R. C., & Campbell, C. S. (2003). Ancient allopolyploid speciation in Geinae (Rosaceae): evidence from nuclear granule-bound starch synthase (GBSSI) gene sequences. *Syst Biol, 52*(3), 374-385. doi:10.1080/10635150390197000

Soltis, P. S., & Soltis, D. E. (2000). The role of genetic and genomic attributes in the success of polyploids. *Proceedings of the National Academy of Sciences of the United States of America, 97*(13), 7051-7057.

Sorenson, A. E. (1986). Seed dispersal by adhesion. *Annual Review of Ecology and Systematics, 17*, 443-463.

Taylor, K. (1997). *Geum Rivale* L. *Journal of Ecology, 85*(5), 721-731.

Ulrey, C., Quintana-Ascencio, P. F., Kauffman, G., Smith, A. B., & Menges, E. S. (2016). Life at the top: Long-term demography, microclimatic refugia, and responses to climate change for a high-elevation southern Appalachian endemic plant. *Biological Conservation, 200*, 80-92. doi:10.1016/j.biocon.2016.05.028

Vandepitte, K., Honnay, O., Jacquemyn, H., & Roldán-Ruiz, I. (2010). Effects of outcrossing in fragmented populations of the primarily selfing forest herb *Geum urbanum*. *Evolutionary Ecology, 24*(6), 1353-1364. doi:10.1007/s10682-010-9395-0

Weakley, A. S. (2015). *Flora of the Southern and Mid-Atlantic States*: University of North Carolina Herbarium, North Carolina Botanical Garden, University of North Carolina, Chapel Hill.

Weppler, T., & Stöcklin, J. (2005). Variation of sexual and clonal reproduction in the alpine *Geum reptans* in contrasting altitudes and successional stages. *Basic and Applied Ecology, 6*(4), 305-316. doi:10.1016/j.baae.2005.03.002

Wiser, S. K. (1994). high-elevation cliffs and outcrops of the Southern Appalachians: vascular plants and biogeography. *Castanea, 59*(2), 85-116.

Yumoto, T. (1986). The ecological pollination syndromes of insect-pollinated plants in an alpine meadow. *Ecological Research, 1*(1), 83-95.

Chapter 2: Census of Occurrences and Establishment of a Demography Plot

Introduction

Monitoring is defined as "the collection of repeated observations or measurements to evaluate changes in condition and progress toward meeting a management objective," (Elzinga, Salzer, Willoughby, & Gibbs, 2009). Resource monitoring is important in land management and conservation for many reasons, including: 1) estimating population size, 2) detecting change in abundance or viability therefore identifying potential problems early, 3) as a tool to determine if management practices are effective, 4) determining habitat requirements for a species and 5) guiding future research questions, (Elzinga, Salzer, & Willoughby, 1998; Menges & Morgan, 1996; Sutherland, 1996). Menges and Morgan (1996) outline three levels of monitoring intensities ranging from low intensity (monitoring distribution of populations), to moderate intensity (monitoring population size and condition), to high intensity (demographic monitoring of individuals).

Most states keep record of the distribution of populations of rare flora, fauna, and their communities through their Heritage Programs. For instance, the North Carolina Natural Heritage Program (NCNHP) collects information on ~31,000 occurrences known as element occurrences (EO) of rare flora, rare fauna, natural communities and animal assemblages across the state of North Carolina (NCNHP, 2018). When these occurrences are visited by professional researchers or partners, information including size of area in which the population occurs, estimates of numbers of individuals, information about phenology, evidence of reproduction, habitat, potential threats, etc. are expected to be submitted. Unfortunately, due to the lack of resources and high numbers of element occurrences some are not updated regularly or information at each occurrence is not collected using the same methods. Because these observations are not collected

consistently, they are treated qualitatively (Elzinga et al., 1998). However, these data are still useful, and the Heritage Program compares previously submitted information about these species or communities to determine management strategies and threat levels.

Priority for more intensive quantitative monitoring and surveys is likely made on those species that have a higher ranking of threat at the state or federal level. Quantitative monitoring of size and condition of populations typically consists of a census of population counts or measurements of a specific attribute for every individual (Elzinga et al., 1998; Menges & Morgan, 1996). When it is not possible to census the entire population, sampling a subset of the population typically occurs. Permanent transects or quadrats are established and numbers of individual or percent cover are collected. These data are used as an estimate for the entire population (Elzinga et al., 1998) and can be compared annually to monitor population changes. However, this method has limitations and does not address variables such as reproduction, survival, or growth as they vary with age or stage class.

Demographic monitoring allows researchers to understand population dynamics of rare species and is appropriate for those at the highest risk of population decline (Menges & Morgan, 1996). Data collected can be used to make projections about population extinction and/or viability and then, utilized by land managers to write informed recovery management plans (Zeigler, Che-Castaldo, & Neel, 2013). Typically, measurements of different attributes of individuals such as age class, flower or seed production and growth measurements are taken annually. In order to properly inform management decisions, the demographic monitoring data should be collected across multiple populations and over many years (15-20) to properly inform the long-term viability of a species (Zeigler et al., 2013).

Population viability analyses typically combine demographic monitoring data with genetic and/or environmental data to produce simulation models which evaluate a plant population's ability to endure (Menges, 2000). For example, high elevation rock outcrop communities provide a cool, moist microclimate that is a refugium for the federally listed, southern Appalachian endemic *Geum radiatum.* Ulrey et al. (2016) compared demographic data of *G. radiatum* to climatic data and modeled scenarios to predict demographic changes in the face of climate change. They found that climate change would affect vital rate, population viability and trigger a decline in population growth (Ulrey et al. 2016).

For rare, economically valuable plants such as *Panax quinquefolium* (American ginseng), demographic monitoring is used in management. In a study in the northern part of its range, low seedling recruitment and slow growth rate of *P. quinquefolium* was observed. Based on this, the authors recommended harvest limits (Charron & Gagnon, 1991). Results of demographic monitoring can be used to increase protections on species that are of economic interest such as permit requirements for harvest, limiting numbers of individuals that can be harvested and time of harvest.

Geum geniculatum is a rare, endemic to the southern Appalachians only occurring on three mountaintops between North Carolina and Tennessee. The species is monitored through the NC Natural Heritage Program (NCNHP) and information about population and subpopulation characteristics: size, viability, threats, habitat, and location are stored in Element Occurrences (EO). *Geum geniculatum* has 28 EOs, 3 of which are parent EOs made up of several sub-EOs. Some of these EOs have not been visited since the 1990s and not all have been visited by the same researchers. There have also been no formal biological studies of *Geum geniculatum.* In 1980, a survey of 12 threatened plant species including this one by Massey and Whitson was

performed and in 1991 an Element Stewardship Abstract was prepared by Shawn Oakley. This abstract contained an overall summary of the species and included research needs and recommendations for management. Because of the length of time since *G. geniculatum*'s last reviews there is a need to update element occurrence data, and work proposed by Oakley should be executed. Between 2018 and 2019 many element occurrences were visited and censused. A long-term demography study was implemented at Population 1in order to begin to address the need to understand life history traits.

Methods

Census 2018

The NC Natural Heritage program organizes the occurrences with Parent EOs consisting of subpopulations or sub-EOs. There is one parent EO for Population 1 (Chapter 1-Figure 1, EO 21) and no subpopulations and one parent EO at Population 2 (EO 26) with 7 subpopulations (Table 1). There are two parent EOs for Population 3: EO 24 and EO 25. Element occurrence 24 is the parent for 2 subpopulation while EO 25 is the parent for 14 sub-EOs (Table 1). In addition, an individual occurrence (EO 31) on United States Forest Service (USFS) land and ~3 individuals are found on private land (EO 32). Using GPS coordinates and GIS polygons supplied by the North Carolina Heritage Program, an attempt was made to look for sub-EOs within each Parent EO.

Table 1. Parent EOs and associated sub-EOs organized by population.

Population	Parent EO	Sub-EO
1	21	
2	26	2, 5, 6, 7, 15, 16, 17
3	24	3, 22
	25	1, 4, 9, 10, 11, 12, 13, 14, 20, 23, 27, 28, 29, 30
	31	
	32	

At sites where individuals were found, flowering stems were counted. A thick herbaceous understory made it difficult to identify all vegetative rosettes, so these were only estimated at some sites. Co-occurring species and any natural or anthropogenic disturbance such as trampling, or herbivory were noted for all EOs. Canopy cover was measured using a forest densiometer (Spherical Crown Densiometer Convex Model A, Forestry Suppliers Inc., Jackson, MS): four measurements were taken, averaged and rounded to the nearest 5%. Comparisons were also made between the NC Heritage records and site observations to describe changes since the last census.

Demography Survey

In July 2019 three long-term demography study plots were constructed at Population 1, which had 4 sub-populations. Two sites, BH and BL, are on a high-elevation grassy bald and the third site, BO, is between a grassy bald and *Cretaegus* forest. In order to locate plants from year to year a permanent baseline was staked using electrical conduit at each plot. Plants were then located by measuring the distance along baseline and distance from the baseline at a 90-degree angle (Figure 1; Elzinga et al., 1998).

Each plant was measured for rosette length and width, number of flowering stems, and flowers per inflorescence, then herbivory or other damage was noted. A template for future monitoring was constructed (Figure 2) and shared with the Estep Lab at Appalachian State University to ensure consistency in future monitoring activities. Data was entered into Excel (version 16.3 © Microsoft 2019) and rosette area and flowering stem per rosette were calculated.

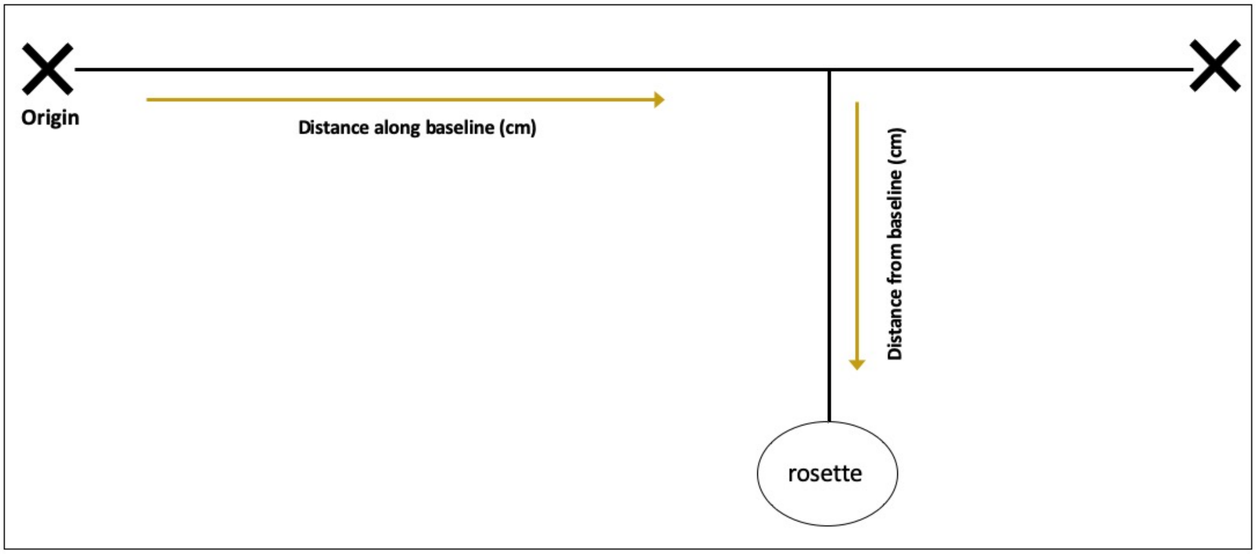

Figure 1. Model for set-up of demography plots of *Geum geniculatum*, adapted from Elzinga et al. (1998).

Name of Observer				Name of Site				Page ___ of ____
Plant ID	Distance along baseline (cm or m)	Distance from baseline (cm or m)	Length (cm)	Width (cm)	# Flowers	# Flowering Stems	Damage	Comments

Measure length and width of rosettes in cm, count number of flowers per inflorescence, note any damage 1 = herbivory, 2 = trampling, 3 = insect damage, 4 = other (note what type of damage if possible)

Figure 2. Sample of demography monitoring form to be used for *Geum geniculatum*.

Results

Summer 2018 Census

During summer 2018, 44.8% (13) of the EOs were visited for *Geum geniculatum* across its range (Table 2). At Population 1 (EO 21), both subpopulations were surveyed. Population 1 is a plant preserve in which the public is not allowed. New subpopulations were found on the grassy bald and along an illegal trail that leads to a housing development adding new subpopulations to this occurrence. At Population 2 (Principal EO 26), 5 sub-EOs (EO 2, EO 5, EO 6, EO 7 and EO 17) were located and surveyed. At Population 3, no plants were found at EOs 20, 24, 28 or 29 (Table 2).

Flowering stems per site ranged from 2 (EO 5) to 307 (EO 4) and the mean across the sites was 84.6. Estimates of vegetative rosettes were made at 8 sub-EOs and ranged between 12 (EO 22) and 283 (EO 7). Canopy cover ranged from 0-5% at grassy bald EOs (EO 21C, 21D, 14) to 90-95% for streambank EO within forests. At ~50% of the sites visited there was trace amounts of browsing. At one site along the Appalachian Trail (EO 1) the plant had been mowed by an unknown source occurred (Table 2).

Demography

Number of plants across the plots ranged from 2 (BL) to 11 (BH). Area of rosettes across the plots ranged from 112 cm ^{2} to 612 cm^2 and the overall mean rosette area was 326.6 cm^2. All of the plants except for 1 was reproductive. Flowering stems per plant ranged from 0 to 3, number of flowers per plant ranged from 6 to 35, and average number of flowers per stem ranged from 6 to 14 while the overall mean number of flowering stems across the plots was 1 and 9 flowers per plant (Table 3). In addition, no herbivory or damage was observed during this first year of data collection.

Table 2. Element occurrences visited during the Summer 2018. If *Geum geniculatum* was found, # of vegetative rosettes, # flowering stems, % sunlight measured, any additional notes and any disturbance observed are listed. * indicates where data was not collected.

Visit Date	Pop	Parent EO	EO	Found	# Vegetative	# Flowering stems	Sunlight	Notes	Trampling	Browsing	Insect Damage	Competition /succession
3-Jul-2018	1	21	21(A)	Yes	0	~70	15%	Interspersed with *Geum canadense*	*	*	*	*
28-Jun-2018	1	21	21(B)	Yes	18	3	25%	*	none	trace	trace	none
3-Jul-2018	1	21	21(C)	Yes	*	3	95%	*	none	none	none	none
3-Jul-2018	1	21	21(D)	Yes	*	22	95%	*	none	none	none	none
13-Jul-2018	2	26	2	Yes	*	20	10%	*	*	*	*	*
20-Jul-2018	2	26	5	Yes	31	2	10%	Next to cliff limiting sunlight	trace	none	none	most
20-Jul-2018	2	26	6	Yes	30	84	*	*	none	trace	none	*
13-Jul-2018	2	26	7	Yes	283	102	10%	*	none	none	none	none
13-Jul-2018	2	26	17	Yes	*	3	*	*	*	*	*	*
10-Jul-2018	3	24	22	Yes	12	*	10%	*	none	none	trace	none
10-Jul-2018	3	24	24	No	*	*	*	*	*	*	*	*
11-Jul-2018	3	25	1	Yes	*	81	20%	Plants mowed down	some	none	none	some
11-Jul-2018	3	25	4	Yes	90	307	10%	*	none	trace	some	none
31-Jul-2018	3	25	10	Yes	*	16	15%	*	none	some	none	none
9-Jul-2018	3	25	14	Yes	0	8	95%	*	none	none	none	none
12-Jul-2018	3	25	20	No	*	*	*	Only *Geum canadense* observed	*	*	*	*
9-Jul-2018	3	25	28	No	*	*	*	*	*	*	*	*
9-Jul-2018	3	25	29	No	*	*	*	*	*	*	*	*
Overall Mean					58	84.6						

Table 3. Data collection for first year of data collection for demography data for *Geum geniculatum* at Population 1.

Plot	Plant ID	Area (cm2)	# Flowers	# of Flowering Stems	Flowers per stem
BL	001	522	15	1	15
BL	002	242	18	2	9
	Total	**764**	**33**	**3**	**11**
	Mean	**382**	**16.5**	**1.5**	**11**
BH	101	384	0	0	0
BH	102	600	6	1	6
BH	103	130	7	1	7
BH	104	320	13	1	13
BH	105	130	6	1	6
BH	106	308	9	1	9
BH	107	612	0	0	0
BH	108	380	8	1	8
BH	109	238	14	1	14
BH	110	272	10	1	10
BH	111	306	35	3	11.7
	Total	**3680**	**108**	**11**	**9.8**
	Mean	**334.5**	**9.8**	**1.0**	**9.8**
BO	201	464	0	0	0
BO	202	240	7	1	7
BO	203	288	0	0	0
BO	204	330	0	0	0
BO	205	112	6	1	6
	Total	**1434**	**13**	**2**	**6.5**
	Mean	**286.8**	**2.6**	**0.4**	**6.5**
	Overall Total	**5878**	**154**	**16**	**9.625**
	Overall Mean	**326.6**	**8.6**	**0.9**	**9.625**

Discussion

Census

Previous reports suggest some populations containing thousands of individuals however the 2018 census revealed lower population sizes, and no plants were found at four EOs. The

inability to locate 3 occurrences could be due to the large polygons recorded by NCHP, difficult to navigate terrain, extirpation at the sites due to encroachment of dense *Rhododendron* sp. thickets, or erosion. Along the Appalachian Trail there were two adjacent EOs. At one occurrence plants were found along the trail, while at the other the only species of *Geum* occurring was *G. canadense,* suggesting earlier misidentifications. Population sizes may be larger than surveyed because the thick herbaceous layer made it difficult to visualize all vegetative rosettes, and the steep terrain made access difficult. Overall, the most robust sub-populations (EO 7 and 4) were those occurring in and along streambanks in northern hardwood forests with only 5-10% sunlight. The sub-population occurring along the heavily used trail in a transition zones between grassy bald and forest (EO 1) and the sub-population occurring in an old roadbed (EO 21A) were also occurring in high numbers. These trails provide similar light requirements to streambanks and seed dispersal at these sites is likely due to humans or animals.

Demography

The plots established at Population 1 will be visited annually, and data will be collected on number of vegetative rosettes, number of flowering stems, number of flowers and rosette size. Additional plants may be tagged to enhance the dataset. The demography monitoring data can be used to perform age/stage class structure analysis (reproductive output, longevity, survival) and demographic structure (percent of individuals within different stage classes; Elzinga et al., 1998). Preliminary results suggest that plants produce 1 flowering stem with ~ 10 flowers per stem, and the mean rosette area is 326.6 cm^2 (Table 2). However, during censusing of other sites flowering stems per plant ranged from 1 to 5, so reproductive output could vary among populations (personal observation). The establishment of additional plots at the other two populations could

enable more accurate determination of long-term species viability. It is critical for conservation management plans to understand population dynamics throughout the whole species, as these can vary between populations (Zeigler et al., 2013). These data will then be able to be combined with the genetic data that has been collected and other environmental data at these sites to perform population viability analyses and determine the persistence or extinction risks of *Geum geniculatum.*

Literature Cited

Charron, D., & Gagnon, D. (1991). The demography of northern populations of *Panax quinquefolium* (American Ginseng). *Journal of Ecology, 79*(2), 431-445.

Elzinga, C. L., Salzer, D. W., & Willoughby, J. W. (1998). *Measuring and Monitoring Plant Populations*: U.S. Bureau of Land Management Papers.

Elzinga, C. L., Salzer, D. W., Willoughby, J. W., & Gibbs, J. P. (2009). *Monitoring Plant and Animal Populations: A Handbook for Field Biologists*: John Wiley & Sons.

Menges, E. S. (2000). Applications of Population Viability Analyses in Plant Conservation. *Ecological Bulletins, 48*, 73-84.

Menges, E. S., & Morgan, D. R. (1996). Three levels of monitoring intensity for rare plant species. *Natural areas Journal : a quarterly publication of the Natural Areas Association, 16*(3), 227-237.

NCNHP. (2018). North Carolina Natural Heritage Program Element Occurences. *Natural Heritage Program.* Retrieved from https://www.ncnhp.org/activities/conservation/natural-heritage-element-occurrences

Oakley, S. (1991). *Element Stewardship Abstract for* Geum geniculatum. The Nature Conservancy. Arlington, VA.

Sutherland, W. J. (Ed.) (1996). *Ecological Census Techniques*. Cambridge, UK: Cambridge University Press.

Ulrey, C., Quintana-Ascencio, P. F., Kauffman, G., Smith, A. B., & Menges, E. S. (2016). Life at the top: Long-term demography, microclimatic refugia, and responses to climate change for a high-elevation southern Appalachian endemic plant. *Biological Conservation, 200*, 80-92. doi:10.1016/j.biocon.2016.05.028

Zeigler, S. L., Che-Castaldo, J. P., & Neel, M. C. (2013). Actual and potential use of population viability analyses in recovery of plant species listed under the U.S. Endangered Species Act. *Conservation Biology, 27*(6), 1265-1278. doi:10.1111/cobi.l2130

Chapter 3: Investigating Insect Visitors via Time-lapse Camera Trapping

Introduction

Genetic variation and high allelic richness is important in plant populations because it provides adaptive capacity in the face of environmental change (Allendorf, Luikart, & Aitkin, 2013; Reed & Frankham, 2003). This variation arises through the process of mutations and is passed to offspring via sexual reproduction. For plant species whose primary reproductive strategy is outcrossing, genetic variation within populations should be high, with rare species displaying lower genetic variation than their more common sister taxa (Cole, 2003; Hamrick & Godt, 1996). In plants, sexual reproduction occurs via pollination; 87.5% of flowering plants worldwide rely on animal-pollination and the majority of this occurs via insect pollination (Ollerton, Winfree, & Tarrant, 2011). The negative consequences of anthropogenic influence on native bees has been acknowledged since the 1970s therefore pollinator decline is a major concern in plant conservation (Tepedino, 1979).

Some plants have evolved other mechanisms to ensure pollination via outcrossing through mixed mating systems including the ability to self-pollinate (self-compatibility) or reproduce asexually. This can be advantageous in that they are able to persist in the absence of pollinators and establish themselves in areas where their natural pollinators are absent (Grossenbacher, Briscoe Runquist, Goldberg, & Brandvain, 2015). The disadvantage of solely relying on self-pollination is that it can lead to inbreeding depression due to accumulation of deleterious alleles (Charlesworth & Willis, 2009). In small plant populations, particularly of rare species or fragmented population such as *G. geniculatum*, cross pollination with genetically variable plants decreases, therefore self-pollination, consanguinity and clonal reproduction have

the potential to become common. For specialist pollinators, if their primary food plant population size drops, the pollinator population also decreases, or can even become extirpated (Kwak, Velterop, & Andel, 1998). Self-pollination in plants can also lead to inbreeding depression, genetic drift leading to decreased fitness, and ultimately extirpation or extinction (Honnay & Bossuyt, 2005; Tanaka, 1997).

Gene flow via pollen transfer between populations is crucial for long-term viability and genetic structure by reducing genetic drift and increasing effective population size (Ellstrand, 1992). Plant population size affects gene flow or exchanging of alleles between populations of plants whose primary reproductive strategy is outcrossing (Ellstrand, 1992). In small populations, potential pollinators should spend less time at each population because of the lower floral density (Ellstrand, 1992). Therefore, interpopulation gene flow should increase as population size decreases (Ellstrand, 1992). This would cause less genetic structuring based on geographic location as long as the distance between populations does not exceed the pollinators' foraging range. Alternatively, when distance between small populations exceeds that of the foraging range there is a barrier to gene flow resulting in distinct genetic structuring between populations.

Breeding systems also affect genetic structure. Plants can rely solely on outcrossing to reproduce; many have a mixed reproduction strategy including self-compatibility and clonal reproduction in order to persist. Species that rely on outcrossing, pollination strategies can also differ from wind-pollinated to animal-pollinated. In terms of population genetic structure and variation, species whose primary reproductive strategy is outcrossing it is expected that effective population sizes will be high as well as within population variation and there will be little genetic structuring among populations, but factors such as pollinator type can vary these results

(Hamrick & Godt, 1996; Loveless & Hamrick, 1984). For instance, in plants whose primary pollinator are small insects, foraging distance will be small, therefore it is expected to have high differentiation between populations. The larger the pollinator, the less differentiation will occur because pollen dispersal increases with foraging distance (Loveless & Hamrick, 1984).

The pollination syndrome, reproductive strategy, and pollen vector for *G. geniculatum* are unknown. *Geum rivale*'s dominant breeding system is outcrossing, and in the British Isles it is pollinated by *Bombus hortorum* and *Apies mellifera* (Ruhsam, Hollingsworth, Squirrell, & Ennos, 2010; Taylor, 1997). *Geum urbanum* is highly self-compatible and predominately visited by syrphid and muscid flies (Ruhsam et al., 2010). Classical ideas of pollinations syndromes where floral traits such as morphology, color, nectar reward and phenology correspond to pollinator type is controversial however, identifying floral traits can still be informative with the addition of other pollination studies (Ollerton et al., 2009; Rosas-Guerrero et al., 2014). Using our understanding of classical pollination syndromes, we can predict potential pollinators for *G. geniculatum* based on the known floral traits of the species. Classical pollination syndromes suggest that the white, campanulate flower suggests that it would be pollinated by insects from the order Coleoptera or the order Diptera (Willmer, 2011). However, additional traits need to be measured in order to predict the pollination syndrome, including odor, presence of nectar guides, nectar abundance, and pollen characteristics.

It is important to note that insect visitation does not necessarily mean pollination. Kearns and Inouye (1993) outline the observations required to distinguish between floral visitors and pollinators: 1) observing pollen being transferred from the visitor to the stigma or, 2) transferred between flowers on a plant or among plants and 3) to confirm fertilization or seed production. A variety of experiments can be done in order to effectively identify pollination. Pollination or

effectiveness can be measured using single visit deposition experiments (King, Ballantyne, Willmer, & Freckleton, 2013). Identification of breeding systems in plants can be important to identify pollinator effectiveness as well as elucidate information about genetic structure. This can be done by setting up pollination treatments for open-pollination, self-pollination, auto-pollination, geitonagamy and xenogamy (Delaplane et al., 2015; Jabis, Ayers, & Allan, 2011; Kearns & Inouye, 1993).

Previous observation has suggested that bumblebees have visited *G. geniculatum* (Oakley, 1991); however, there have been no formal studies to support this observation. In order to confirm previous observations and to identify any additional insect visitors a time-lapse camera trapping study was performed. This is the first step in order to identify the pollen vector for *G. geniculatum* as well as the breeding system. Combining data collected from insect visitor and pollinator studies with the results of a genetic diversity study, can help to elucidate what forces are acting to structure populations and the species as a whole.

Methods

Floral Visitor Survey

Photography has been shown to be an effective non-invasive method to document insect diversity (McCullough, Worthington, & Paradise, 2013). Time-lapse photography has taken the use of photography a step further in being able to identify nearly all flower visitations as well as identify peak times of day for visitations (Edwards, Smith, & McEntee, 2015). Methods for the time-lapse survey of *Geum geniculatum* were adapted from Edwards et al. (2015). *Geum geniculatum* flowers between late-June and August (Weakley, 2015); therefore, the camera was set up in July 2018 at the three different mountains on which the species occurs. A Brinno HD

TLC 200 Pro (Figure 1, Brinno, Taipei City, Taiwan) with weatherproof housing was set up to record 20 frames per second at 5 second intervals for 12-hour periods (0600 to 2100) at each of these locations. The camera was set up to record in a grassy bald habitat in Population 1, Populations 2 was in a boulderfield forest, and Population 3 occurred along a streambank.

Figure 1. Brinno HD TLC 200 Pro and *Geum geniculatum* at Population 3 in July 2018.

No floral visitors were observed in 2018 therefore cameras were set up again at shorter intervals (3 seconds) at 20 frames per second for 12-hour periods at Population 1 from 12 June – 16 June 2019 and at Population 3 from 21 June –23 June 2019. Videos were reviewed in Adobe Premier Pro CC 2019. Floral visitation was identified as any insect landing on the inflorescence (Edwards et al., 2015). Time spent within the frame, number of flowers visited and total number of flowers in bud were also recorded. Insects were identified to the lowest order of classification

possible, typically family or genus, using a field guide (Borror & White, 1970), and photos were sent to Ray Williams in the Department of Biology at Appalachian State University for identity verification.

Results

Floral Visitor Survey

In July 2018 the camera was set up for 8 days, during which it recorded for a total of 84 hours 54 minutes and 51 seconds across the three populations (Table 1). It was set up from 3 July 2018 – 6 July 2018 for 40 hours 22 minutes and 4 seconds at Population 1. It was set up from 17 July 2018 to 19 July 2018 for 34 hours, 12 minutes and 20 seconds at Population 2. At Population 3 the camera was set up for 6 hours 20 minutes and 16 seconds on 6 July 2018. While some Syrphidae insects were documented in the recordings (Figure 2), no insect came in contact with the flowers; therefore, they could not be considered floral visitors.

Table 1. Table indicating each population visited in 2018, including date, start and end of recording and total number of hours recorded per day, total number of hours recorded per site, and total number of hours across all sites.

Population	Date	Start Time	End Time	Hours Recorded
1	7/3/18	10:37:56	21:00:00	10:22:04
1	7/4/18	6:00:00	21:00:00	15:00:00
1	7/5/18	6:00:00	21:00:00	15:00:00
1	7/6/18	6:00:00	10:48:11	4:48:11
			Total	44:22:15
3	7/11/18	10:27:22	16:47:38	6:20:16
			Total	6:20:16
2	7/17/18	11:41:21	21:00:00	9:18:39
2	7/18/18	6:00:00	21:00:00	15:00:00
2	7/19/18	6:00:00	15:53:41	9:53:41
			Total	34:12:20
			Overall Total	84:54:51

Figure 2. Photograph taken on 11 July 2018 at Population 2 showing a Syrphideae in the frame with *Geum geniculatum.* There was no capture of the insect coming in contact with the flower.

Because of low visitation in 2018 the camera was set out earlier in the blooming season in summer 2019. At Population 1 the camera was set up to record two flowering stems and 12 flowers in the frame. The camera recorded a total of 45 hours, 46 minutes and 28 seconds across 4 days (Table 2). There was a total of 142 visitations to 327 flowers, with a mean 1.9 flowers per visit across the 4 days the camera was installed. There was 0 visitation the first day the camera was set up. The second day only 5 visitations to 15 flowers occurred. The third day, 65 visitations to 134 flowers occurred. The last day of filming, 72 visitations were recorded to 178 flowers (Table 2). Average time per visit was 24 seconds and average time per visit per day ranged between 20 seconds and 29 seconds. All of the visitations at Population 1 were by *Bombus* sp. (Figure 3).

Figure 3. Time-lapse photo of *Bombus* sp. visiting flower of *Geum geniculatum* at Population 1, a grassy bald habitat on 15 June 2019 at 13:17:21.

The camera was also set up at Population 3 and recorded 27 hours 38 minutes and 45 seconds over a three-day period (Table 2). The camera was focused on one flowering stem with 6 flowers. Here, there were three dominant visitors recorded at this site: *Bombus* sp. (Figure 4), a Syrphid fly (Figure 5) and an unidentifiable insect (Figure 6). At population 3, 4 visitations and 6 flowers were visited by *Bombus* sp. on day 1. On day 2, only one visitation at 1 flower occurred for 12 seconds by a *Bombus* sp.. On the last day of filming there were a total of 18 visitations to 27 flowers (Table 2). Of the 18 visits, 5 were from *Bombus* sp., 2 insects were members of the Family Syrphidae and 11 were by the unidentifiable insect. Visitations lasted between 0 sec and 4 min and 48 sec with the mean visitation time, 1 minute and 4 seconds and average time visited per flower, 42 sec. The longest visitations were by the unknown species.

Figure 4. Time-lapse photo of *Bombus* sp. visiting a flower of *Geum geniculatum* at Population 3 along a streambank on 23 June 2019 at 9:54:58.

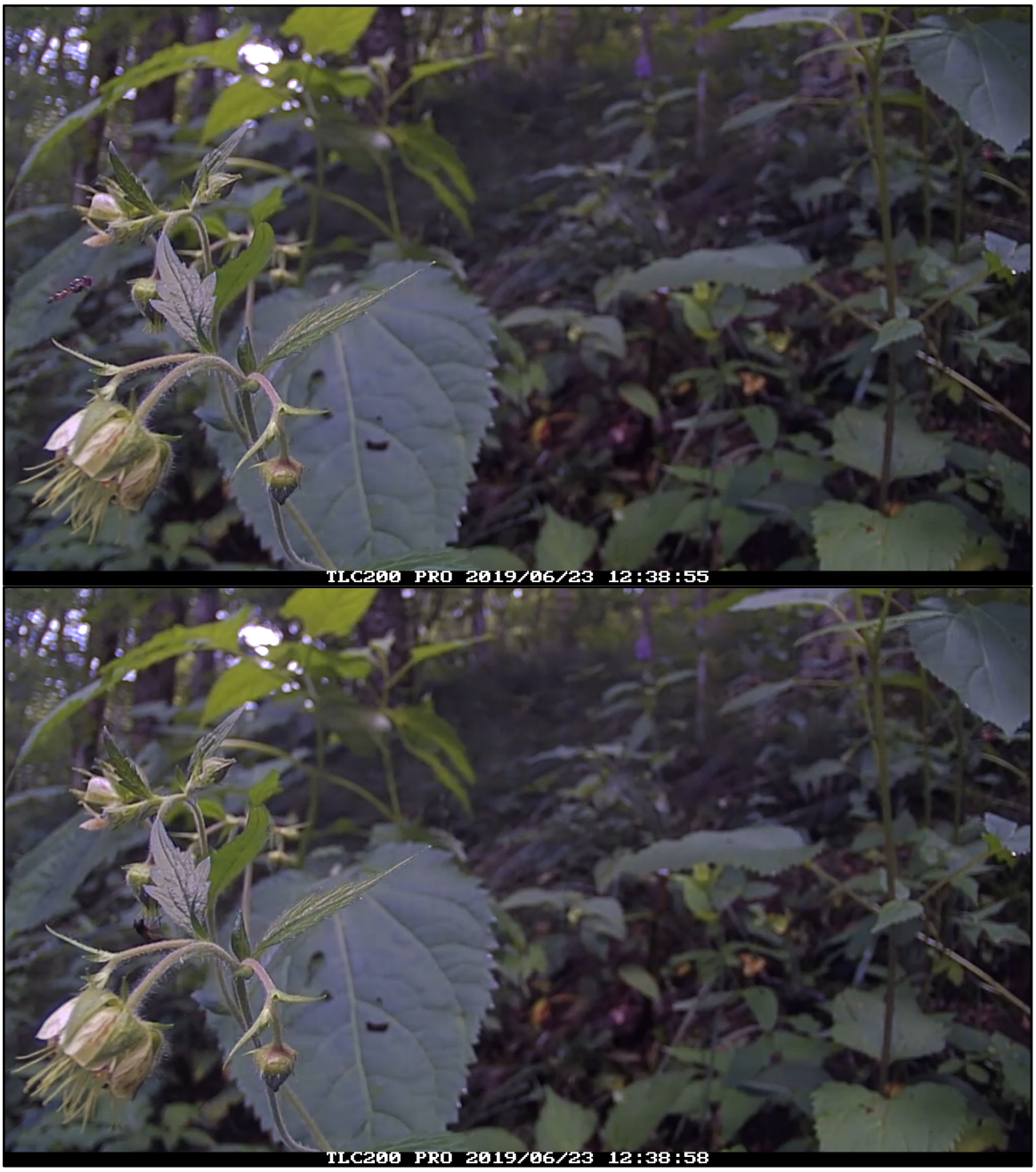

Figure 5. Syrphid fly visiting *Geum geniculatum* on 23 June 2019 at 12:38:55. The top image is the fly approaching the flower, and the second is contact with the flower.

Figure 6. Unknown insect visitor to *Geum geniculatum* at Population 3 on 23 June 2019 at 13:46:04. Difficulty to see wing venation in photograph makes it difficult to identify type of visitor (personal correspondence with Ray Williams).

Discussion

In July 2018 the camera was set up for a total of 8 days across the three mountains. There were no floral visitors during this year's observation period. Flowering typically occurs from late June to August; however, flowers may have been beginning to set fruit hence the lack of floral visitors. Factors that also could have affected the lack of floral visitors include the angle the camera was positioned to record the flowers, for instance, at Population 1 there was a glare within the frame that could have affected insect visualization. There was also concern about how long the cameras would record, so initially, cameras were set to take images at 5 second intervals rather than 3 seconds, as recommended in Edwards et al. (2015) which could have attributed to less insects recorded within the frame.

In order to troubleshoot this, the following year images were taken at 3 second intervals and set out at the beginning of the bloom season. Plants had flowers in all stages, from bud to bloom, during this period. Population 1 had a greater number of visitors than Population 3,

however only bumblebees visited at Population 1. In day 2 of recording only 5 visitors were documented which, far fewer than in the following two days. The reason for this difference on this day is unclear compared to the succeeding two days as weather (temperature, cloud cover, etc.) during these days appeared similar.

Population 3 had less visitation over the duration of the survey but had a wider variety of visitors than Population 1. On 22 June 2019 an error with the camera occurred only, recording until 11:49:06, therefore decreasing the amounts of visitations observed. Higher mean visitation times at Population 3 were likely due to the differing insect species that were observed. Different functional groups of insects have been found to have varying rates of visitation times. For example, bumblebees have been shown to visit more flowers per minute (11.5 per minute) than Syrphid flies (4.8 per minute; Couvillon et al., 2015). Higher visitations at Population 1 are likely due to the habitat. High elevation grassy balds are characterized by minimal shrubs and many showy perennial flowers such as *Lilium grayi*, *Rudbeckia laciniata, Solidago glomerata*, *Angelica triquinata*, and *Packera schweinitziana* which attract many pollinators. At Population 3 the camera was set along a streambank in a hardwood forest where sunlight was limited and there were no attractive herbs in flower. The only herb in flower nearby was *Laportea canadense* likely contributing to the lack of floral visitors.

It is interesting that the dominant visitor to *Geum geniculatum* was *Bombus* sp. as, its pollination syndrome based on floral traits for the species suggests fly (Order Diptera) or beetle (Order Coleoptera) pollination. Plants that are adapted to pollination by *Bombus* sp. typically display a pollination syndrome of large, bright, flowers with nectar rewards (Anderson, Anderson, & Houseman, 2002; Willmer, 2011). However, *Geum rivale,* its closest sister taxa has similar floral morphology and is pollinated by bumblebees. Visitation may also be a

byproduct of bees haphazardly visiting all blooming plants within their foraging range. Bumblebees forage as close to their nest as possible, however in the lack of suitable pollen sources have the ability to travel up to 10 km for food (Goulson, 2010).

These initial surveys provide a baseline for identifying insect visitors and potential pollinators for the species. However, a full pollination study is recommended for the species to confirm insect visitors vs. insect pollinators. North American bumblebees have been shown to be experiencing a decline compared to historic records (Cameron et al., 2011). It is important to determine the pollinator for *G. geniculatum*, if it is *Bombus* sp. additional conservation efforts might need to be addressed.

Breeding systems can be suggested based on genetic analysis; however, breeding experiments should be conducted to confirm the species' reproductive strategy. Seed storage and germination might be similar to its relative, *Geum macrophyllum*. Guerrant and Raven (1998) found that *G. macrophyllum* had higher germination rate using fresh seed than dried seed. These results imply that dry storage is not suitable for this species. If germination is similar in *G. geniculatum* it might more appropriate to store *G. geniclutum* in a living seed bank, such as a nursery rather than a dried storage facility; therefore, germination studies will prove useful.

Table 1. Summary of data collected for time-lapse video survey during year 2 at populations 1 from 6/12/19 to 6/15/19 and population 3 from 6/21/19 to 6/23/19 including time of camera recording, total hours recorded, # of visitations, # of flowers the insect came in contact with, average number of flowers contacted per visit, time visited, and average time per visit.

Population	Date	Start Time	End Time	Hours Recorded	# Visitations	# Flowers	Average Flowers per Visit	Time Visited	Average Time Visited	Time Per Flower
1	12-Jun-2019	16:33:29	21:00:00	4:26:31	0	.	.	.	.	.
1	13-Jun-2019	6:00:00	21:00:00	15:00:00	5	15	3	0:02:24	0:00:29	0:00:10
1	14-Jun-2019	6:00:00	21:00:00	15:00:00	65	134	2.1	0:25:30	0:00:24	0:00:11
1	15-Jun-2019	6:00:00	17:19:57	11:19:57	72	178	2.5	0:23:44	0:00:20	0:00:08
			Mean		35.5	81.8	1.9	0:12:55	0:00:24	0:00:10
			Total	45:46:28	142	327	2.3	0:51:38	0:00:22	0:00:09
3	21-Jun-2019	12:59:58	21:00:00	8:00:02	4	6	1.5	0:00:39	0:00:10	0:00:07
3	22-Jun-2019	6:00:00	11:49:06	5:49:06	1	1	1	0:00:12	0:00:12	0:00:12
3	23-Jun-2019	6:00:00	19:49:37	13:49:37	18	27	1.5	0:23:45	0:01:19	0:00:53
			Mean		7.7	11.3	1.3	0:08:12	0:00:34	0:00:24
			Total	27:38:45	23	34	1.5	0:23:57	0:01:04	0:00:42
			Overall Mean		23.6	51.6	1.7	0:10:53	0:00:25	0:00:17
			Overall Total	73:25:13	165	361	3.8	1:15:35	0:00:43	0:00:25

Literature Cited

Allendorf, F. W., Luikart, G., & Aitkin, S. N. (2013). *Conservation and the Genetics of Populations* (2nd ed.). Oxford: Wiley-Blackwell

Anderson, R. C., Anderson, M. R., & Houseman, G. (2002). Wild American Ginseng. *Native Plants Journal (Indiana University Press), 3*(2), 93-105.

Borror, D. J., & White, R. E. (1970). *A Field Guide to the Insects of America North of Mexico*. New York, NY: Houghton Mifflin Company.

Cameron, S. A., Lozier, J. D., Strange, J. P., Koch, J. B., Cordes, N., Solter, L. F., & Griswold, T. L. (2011). Patterns of widespread decline in North American bumble bees. *Proc Natl Acad Sci U S A, 108*(2), 662-667. doi:10.1073/pnas.1014743108

Charlesworth, D., & Willis, J. H. (2009). The genetics of inbreeding depression. *Nat Rev Genet, 10*(11), 783-796. doi:10.1038/nrg2664

Cole, C. T. (2003). Genetic Variation in rare and common plants. *Annual Review of Ecology, Evolution, and Systematics, 34*(1), 213-237. doi:10.1146/annurev.ecolsys.34.030102.151717

Couvillon, M. J., Walter, C. M., Blows, E. M., Czaczkes, T. J., Alton, K. L., & Ratnieks, F. L. W. (2015). Busy bees: variation in insect flower-visiting rates across multiple plant species. *Psyche: A Journal of Entomology, 2015*, 1-7. doi:10.1155/2015/134630

Delaplane, K. S., Dag, A., Danka, R. G., Freitas, B. M., Garibaldi, L. A., Goodwin, R. M., & Hormaza, J. I. (2015). Standard methods for pollination research withApis mellifera. *Journal of Apicultural Research, 52*(4), 1-28. doi:10.3896/ibra.1.52.4.12

Edwards, J., Smith, G. P., & McEntee, M. H. F. (2015). Long-term time-lapse video provides near complete records of floral visitation. *Journal of Pollination Ecology, 16*(13), 91-100.

Ellstrand, N. C. (1992). Gene flow by pollen: implications for plant conservation genetics. *Oikos, 63*(1), 77-86.

Goulson, D. (2010). *Bumblebees : Behaviour, Ecology, and Conservation*. New York NY: Oxford University Press.

Grossenbacher, D., Briscoe Runquist, R., Goldberg, E. E., & Brandvain, Y. (2015). Geographic range size is predicted by plant mating system. *Ecol Lett, 18*(7), 706-713. doi:10.1111/ele.12449

Hamrick, J. L., & Godt, M. J. (1996). Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions: Biological Sciences, 351*(1345), 1291-1298.

Honnay, O., & Bossuyt, B. (2005). Prolonged clonal growth: escape route or route to extinction? *Oikos, 108*(2), 427-432.

Jabis, M. D., Ayers, T. J., & Allan, G. J. (2011). Pollinator-mediated gene flow fosters genetic variability in a narrow alpine endemic, *Abronia alpina* (Nyctaginaceae). *Am J Bot, 98*(10), 1583-1594. doi:10.3732/ajb.1000515

Kearns, C. A., & Inouye, D. W. (1993). *Techniques for Pollination Biologists*. Niwot, Colorado: University Press of Colorado.

King, C., Ballantyne, G., Willmer, P. G., & Freckleton, R. (2013). Why flower visitation is a poor proxy for pollination: measuring single-visit pollen deposition, with implications for pollination networks and conservation. *Methods in Ecology and Evolution, 4*(9), 811-818. doi:10.1111/2041-210x.12074

Kwak, M. M., Velterop, O., & Andel, J. v. (1998). Pollen and gene flow in fragmented habitats. *Applied Vegetation Science, 1*(1), 37-54.

Loveless, M. D., & Hamrick, J. L. (1984). Ecological determinants of genetic structure in plant populations. *Annual Review of Ecology and Systematics, 15*, 65-95.

McCullough, C., Worthington, C., & Paradise, C. J. (2013). Using digital macrophotography to measure biodiversity, identify insects, and enhance outreach and education. *American Entomologist, 59*(3), 176-182. doi:https://doi.org/10.1093/ae/59.3.176

Oakley, S. (1991). *Element Stewardship Abstract for* Geum geniculatum. The Nature Conservancy. Arlington, VA.

Ollerton, J., Alarcon, R., Waser, N. M., Price, M. V., Watts, S., Cranmer, L., Rotenberry, J. (2009). A global test of the pollination syndrome hypothesis. *Ann Bot, 103*(9), 1471-1480. doi:10.1093/aob/mcp031

Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos, 120*(3), 321-326. doi:10.1111/j.1600-0706.2010.18644.x

Reed, D. H., & Frankham, R. (2003). Correlation between Fitness and Genetic Diversity. *Conservation Biology, 17*(1), 230-237.

Rosas-Guerrero, V., Aguilar, R., Marten-Rodriguez, S., Ashworth, L., Lopezaraiza-Mikel, M., Bastida, J. M., & Quesada, M. (2014). A quantitative review of pollination syndromes: do floral traits predict effective pollinators? *Ecol Lett, 17*(3), 388-400. doi:10.1111/ele.12224

Ruhsam, M., Hollingsworth, P. M., Squirrell, J., & Ennos, R. A. (2010). Significant differences in outcrossing rate, self-incompatibility, and inbreeding depression between two widely hybridizing species of *Geum*. *Biological Journal of the Linnean Society, 101*(4), 977-990.

Tanaka, Y. (1997). Extinction of populations due to inbreeding depression with demographic disturbances. *Researches on Population Ecology, 39*(1), 57-66.

Taylor, K. (1997). Geum Rivale L. *Journal of Ecology, 85*(5), 721-731.

Tepedino, V. J. (1979). The importance of bees and other insect pollinators in maintaining floral species composition. *Great Basin Naturalist Memoirs, 3*, 139-150.

Weakley, A. S. (2015). *Flora of the Southern and Mid-Atlantic States*: University of North Carolina Herbarium, North Carolina Botanical Garden, University of North Carolina, Chapel Hill.

Willmer, P. (2011). *Pollination and Floral Ecology*. Princeton NJ: Princeton University Press.

Chapter 4

Genetic Diversity and Population Structure of the Forgotten *Geum*, *Geum geniculatum* Michaux.

(Article submitted to Castanea)

Marietta Day Shattelroe, Matt C. Estep
Department of Biology, Appalachian State University, 572 Rivers Street, Boone NC, 28607-2027
shattelroemd@appstate.edu, estepmc@appstate.edu

Abstract: *Geum geniculatum* Michx. (Roseaceae), bent avens, is a perennial herb restricted to the high elevations of three mountaintops near the North Carolina/Tennessee border. Although geographically restricted, occurrences on these mountaintops can have up to hundreds and occasionally thousands of individuals. Because of its limited geographic distribution and affinity for high elevations, it is thought to belong to a group of plants endemic to the southern Appalachians that are considered post-Pleistocene relicts including its charismatic cousin *Geum radiatum*. Thorough surveys have been performed for *G. geniculatum;* however, some sites have not been visited in over a decade and formal biological studies are lacking. To understand genetic variation and connectivity within the species and between populations*,* a population genetics study was performed using 14 microsatellite markers. Individuals were sampled from the three mountains where it occurs and genotyped using previously developed microsatellite markers for other *Geum* species. Results of analysis conclude that *G. geniculatum* displays high genetic diversity, and each mountain is acting as three highly structured metapopulations with moderate genetic differentiation between them. Based on the high numbers of private alleles and bottleneck test; genetic drift is driving structure and differentiation among meta populations. In addition, there is no signs of inbreeding within the species.

Introduction

During the Pleistocene epoch, the Laurentide Ice Sheet covered the majority of North America as far south as the 40th parallel north (Delcourt and Delcourt 1987). Thus, the majority of the southeastern United States remained unglaciated. This allowed for the persistence and refugia of many species that were unable to survive in northern latitudes (Russell et al. 2009). During the retreat of the ice sheet during the Last Glacial Maximum, species had two options: migrate north to higher latitudes or remain in refugia at higher elevation regions where habitat remained suitable. These multidirectional migrations created a mosaic of disjunct distributions of species between southern and northern North America as well as island populations at high elevations where climate remained favorable (Delcourt and Delcourt 1987, Wiser 1994, Godt et al. 1996). Additionally, these species became isolated at high elevations, possibly restricting gene flow between populations and allowing for speciation to occur (Delcourt and Delcourt 1988, Godt et al. 1996). For example, spruce-fir forest was once contiguous in the southeast during the Pleistocene but now only exists in relict populations at the highest altitudes in the southern Appalachians (Cogbill and White 1991, Delcourt and Delcourt 1998).

Population genetic studies using allozymes have supported this glacial refugia hypothesis in high elevation plant relict species of *Geum radiatum*, *Carex misera, Trichophorum cespitosum,* and *Calamagrostis cainii* (Godt et al. 1996). All species exhibited low genetic diversity and moderate levels of differentiation, likely related to their small populations and restriction to gene flow due to retraction of suitable habitat since the last ice age. The glacial refugia hypothesis in the Southeastern United States has also been supported in phylogeographic analysis in species such as *Trillium cuneatum* and millipedes in the genus *Narceus* (Gonzales et al. 2008, Walker et al. 2009).

Geum geniculatum Michaux, bent avens, is a rare, perennial herb endemic to high elevations (>1200 m) in the southern Appalachians. It is thought to be a relict species along with other rare species including *Geum radiatum*, *Liatris helleri, Solidago spithamea* and *Calamagrostis cainii (Wiser 1994)*. It is an erect plant growing to almost a meter in height, with a basal rosette of varying leaf shape: trifoliate, pinnately compound, or simple. While the cauline leaves on the flowering stems have varying leaf shape from simple to trifoliate (Oakley 1991). It is characterized by a green to dark purple campanulate or bell-shaped calyx and flowers that nod at anthesis. The flowers are white and can be distinguished from the species that share the same range, *Geum canadense* and *G. virginianum,* not only by calyx color but also by the nodding flowers at anthesis (Oakley 1991, Weakley 2015).

This species occupies grassy balds, streambanks, and seepy boulderfield forests, as well as trail sides on three mountains between North Carolina and Tennessee (Oakley 1991, Weakley 2015). Because of its rare status; population locations are masked for protection (Figure 1). As the crow flies Population 1 and Population 2 are separated by ~23 km, Population 2 and Population 3 by ~27 km and Population 1 and Population 3 by ~41.5 km (Google Earth 2019). Its heterogeneous distribution within this area is due to historic climactic changes of warming and drying periods during the Pleistocene where the moist peaks of high elevations acted as refuge for the species (Weakley 2015).

Its closest relative, *Geum rivale*, is thought to be one of the northern disjunct species previously described. It is a circumboreal species, with a cosmopolitan distribution occurring as far south as central West Virginia (Taylor 1997, Weakley 2015). The genus *Geum* is in the Roseaceae and comprised of 40 to 60 species worldwide, with 14 species in the southeastern United States (Weakley 2015). Plants of the genus *Geum* have a number of species that are both

widespread and relicts that have likely persisted since the Last Glacial Maximum. In the southeastern United States, there are two *Geum* species that are thought to be Pleistocene relict species: *G. radiatum* and *G. geniculaum.* Other known relict species of *Geum* include *G.peckii* occupying the White Mountains of New Hampshire which is sister taxa to *G. radiatum* and *G. bulgaricum* in the Balkan Peninsula (Petrova et al. 2011, Weakley 2015). There have been no formal studies to determine ploidy level for *G. geniculatum* (Smedmark and Eriksson 2002). However, thorough cytogenetic studies by Raynor (Raynor 1952) and Gajewski (Gajewski 1957) have shown that the majority of species within the genus *Geum* are hexaploid (2n = 6x = 42), suggesting *G. geniculatum* is also an hexaploid.

Both *G. geniculatum* and *G. radiatum* share an affinity for high elevations between North Carolina and Tennessee yet their habitat and morphology are quite different. *G. radiatum* prefers sunny, open rock outcrops above 5000 ft in elevation. It has also been listed as endangered since 1990 (NatureServe 2018). Due to its protected status, extensive monitoring has been conducted on it as well as a number of historic augmentations (Ulrey et al. 2016). Two population genetics studies have also been conducted on *G. radiatum*. The first was an allozyme study by Godt and Hamrick (1996) which found low genetic diversity using Nei's genetic diversity index (H_s = 0.119 among populations) and low to moderate differentiation among populations (G_{st} = 0.191, Godt et al. 1996). More recently, a study using microsatellite data using 8 microsatellite loci across the extent of the range (14 populations) concluded high allelic richness across the species (141 across all loci, average per locus) and high observed heterozygosity (mean Ho = 0.635). This high genetic diversity could be due to its hexaploid nature. There was low to moderate differentiation between populations (F_{st} = 0.022 – 0.126) and STRUCTURE analysis suggested 4 distinct clusters separated throughout its geographic range (Hay et al. 2019).

Even though *G. geniculatum*'s range is more restricted than that of *G. radiatum* it does not have any federal protection status but is a federal species of concern (FSC) and likely because of this, has had little biological research and no formal studies have been conducted on it. States and government agencies must prioritize conservation efforts, and this is generally accomplished with a ranking system. *Geum geniculatum* is globally ranked as imperiled (G2) or at high risk for extinction due to its limited range. In Tennessee, it is a state ranked critically imperiled species (S1) and listed as endangered. In North Carolina it is state ranked critically imperiled/imperiled (S1S2) and state listed as a species of concern-vulnerable (SC-V); (Robinson and Finnegan 2016, NatureServe 2018).

The North Carolina Heritage Program collects information on ~31,000 occurrences known as element occurrences (EO) of rare flora, rare fauna, natural communities and animal assemblages across the state of North Carolina (NHP) including *G. geniculatum*. Information about these occurrences are submitted by professional researchers and partnerships. *Geum geniculatum* has 32 element occurrences. There is one EO for population 1 (EO 21), one principal EO at Population 2 (EO 26) consisting of 7 subpopulations (EO 2, EO 5, EO 6, EO 7, EO 15, EO 16, EO 17). There are two parent EOs for Population 3: EO 24 and EO 25. Element occurrence 24 is the principal for 2 subpopulations: EO 3, EO 22. Element occurrence 25 is the principal for 14 subpopulations: EO 1, EO 4, EO 9, EO 10, EO 11, EO 12, EO 13, EO 14, EO 20, EO 23, EO 27, EO 28, EO 29, EO 30.

In 1980 Massey and Whitson performed a survey of the species and in 1991 an Element Stewardship Abstract was outlined for the species by Shawn Oakley which laid out research needs including pollination biology, population demography, and genetic diversity. In an effort to address these needs, a population genetic study was conducted using microsatellite markers

previously developed for *G. urbanum* and *G. reptans* to understand gene diversity, gene flow and population structure of the species (Arens et al. 2004, Hamann et al. 2014). Microsatellite markers are a codominant marker system of tandemly arrayed repeats which can be polymorphic between individuals due to their instability and mutation frequency (Vieira et al. 2016). Co-dominant markers are preferred for population genetics because they directly measure heterozygosity due to their ability to amplify alleles at each locus (Allendorf et al. 2013).

The populations occur in large populations sizes (hundreds to thousands), only occur on three mountains above 1200 m and are separated by distances that could impede gene flow therefore we predict that the mountains are each acting as metapopulations (NCNHP 2018). It is anticipated, like *G. radiatum,* that *G. geniculatum* will have high genetic diversity (allelic richness and heterozyosity) due to its polyploid nature and relatively high numbers of individuals in populations (populations >1000, NCNHP 2018). It is also expected that if *G. geniculatum* is a post-Pleistocene relict there will be evidence of the shared ancestry or relatedness indicating the populations were once connected. The genetic structure, allelic and genetic diversity elucidated from this study will provide land managers with information that can be used to inform future management strategies for the species.

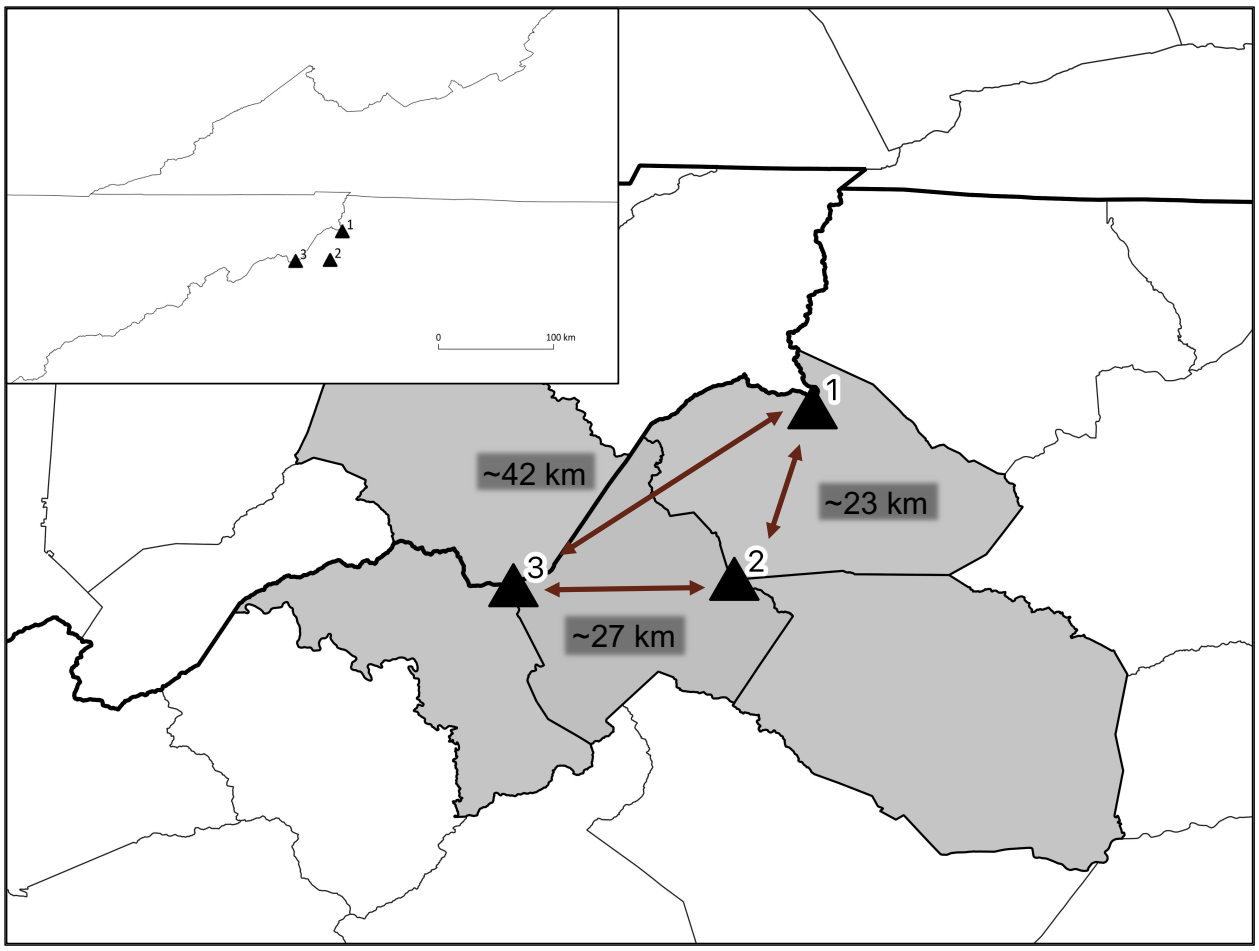

Figure 1. Map indicating *Geum geniculatum* population locations in North Carolina and Tennessee.

Methods

Tissue Collection. Permits were obtained to non-destructively collect leaf tissue sample from the U.S. Forest Service (#APP1164-01), National Park Service (#BLRI-2018-SCI-0010), North Carolina Department of Agriculture Plant Conservation Program (#598), NC Chapter of the Nature Conservancy (Permit issued 9 Feb 2018), Southern Appalachian Highlands Conservancy (#SAHC-001-2018), North Carolina Division of Parks and Recreation (#2018_0200) and the Tennessee Department of Environment and Conservation (#2018-009). Leaf tissue samples were non-destructively collected from ~30 individuals from each element occurrence visited during

the summer of 2018 and stored on silica gel in cryotubes in the field and placed in a -80°C freezer for long-term storage.

DNA Extractions and Genotyping. All leaf tissue samples collected were organized and combined based on mountain peak they occur (Population 1, Population 2, Population 3) and 32 leaf tissue samples from each mountain were randomly selected independent of sub-EO from which they were collected (n = 32 per population; Hale et al. 2012). Leaf tissue was ground using a micropestle and sterile sand and DNA was extracted using a modified CTAB method (Doyle and Doyle 1987). DNA quality and quantity was assessed on an 1% TBE agarose gel and using a Nanodrop 1000 Spectrophotometer (Thermofisher Scientific, Wilmington, DE, USA). DNA was diluted to 30 ng/μL and polymerase chain reaction (PCR) was run using a total of 14 microsatellite markers modified to include a 5' M13 tag (5'-CACGACGTTGTAAAACGAC-3') (Schuelke 2000). These included: 4 microsatellite markers (7389, 11534, 13198, and 14769) developed for *Geum reptans* (Hamann et al. 2014) and 10 microsatellite markers (WGU1-33, WGU2-10, WGU2-28, WGU2-48, WGU3-15, WGU5-11, WGU6-1, WGU6-23, WGU6-5) developed for *G. urbanum* (Table 1; Arens et al. 2004). PCR reactions were prepared in 10 μL volumes consisting of 1x GoTaq Flexi Buffer, 2.5 mM $MgCl_2$, 800 μM dNTPs, 0.5 μM of reverse primer, 0.25 μM of tagged forward primer, 0.25 μM of a M13 fluorescent labeled primer (FAM, VIC, NED, or PET; Invitrogen, Carlsbad, CA, USA) , 0.5 units of GoTaq Flexi DNA Polymerase (Promega Corporation, Madison, Wisconsin, USA), and ~30 ng of DNA. PCR reactions were run using published thermocycler conditions dependent on the marker utilized on an Eppendorf Mastercycler thermocycler (Eppendorf, Hauppauge, New York, USA).

Four PCR products with differing fluorescent tags (VIC, FAM, NED, PET; Invitrogen, Carlsbad, CA, USA) were multiplexed and combined with HI-DI and a GeneScan Liz 500 size standard (Invitrogen, Carlsbad, CA, USA) and sent to Georgia Genomics Facility (Athens, GA, USA) for fragment separation. Resulting chromatograms were assessed and scored in Geneious 9.1 with the Microsatellite Plug-in (Kearse 2012).

Data Analysis. Allele frequency was calculated using the package 'polysat' for R program (Clark & Schreier 2017; R Core Team 2018). Diversity statistics including number of alleles, allelic richness and multi-locus genotypes (MLG) were calculated in the package 'poppr' for R program (Kamvar et al. 2014; Kamvar et al. 2015; R Core Team 2018). Expected heterozygosity, observed heterozygosity as well as inbreeding coefficients (G_{IS} and F_{IS}) with 999 Monte Carlo permutations to test significance were calculated using GENODIVE Version 3.01 (Meirmans and Tienderen 2004). Measures of population differentiation Wright's F_{st}, was calculated using the package 'polysat' (Clark and Schreier 2017) with 1000 bootstrap values and pairwise Nei's G'_{ST} and Jost's D (Jost 2008) were calculated using GENODIVE Version 3.01 (Meirmans and Tienderen 2004).

A Discriminant analysis of principal components was run using the 'adegenet' package for R program (Jombart 2008, R Core Team 2018). A minimum spanning network using MLG and Bruvo's distance as well as a neighbor-joining tree using Bruvo's distance was constructed using the package 'poppr' (Bruvo et al. 2004, Kamvar et al. 2014, Kamvar et al. 2015).

Clustering analysis was run using STRUCTURE (Pritchard et al. 2000). To determine the ΔK values, parameters were set to 50,000 burn-ins with a Monte Carlo Markov Chain (MCMC) of 500,000, run on K-values 1- 25 and 5 iterations per K under an admixture model and

correlated allele frequency model. Results were imported into STRUCTURE Harvester (Earl and vonHoldt 2011) and run under the Evanno method (Evanno et al. 2005). STRUCTURE was run again with the resulting ΔK value of 3 with 10,000 burn-ins and 100,000 repetitions and the Q-matrix was graphed using STRUCTURE Plot (Ramasamy et al. 2014).

An M ratio test was performed to investigate the possibility of a genetic bottleneck. An M-Ratio (M) is used to compare the total number allleles (*k*) to the allele size ranges (*r*) can be interpreted to identify a recent bottleneck (Garza and Williamson 2001). One monomorphic marker was removed from analysis (WGU6-5). The M ratio was calculated per locus within populations in R package 'strataG' (Archer et al. 2016) then averaged across populations. The M critical value (M_c) was calculated using the script "Critical_M" (obtained at https://swfsc.noaa.gov/textblock.aspx?Division=FED&id=3298) under parameters suggested by Garza and Williamson for the two-phase mutation model and theta (calculated in 'strataG').

Results

Genetic Diversity. Eighty-nine individuals (N=89) of *Geum geniculatum* were successfully genotyped across 14 microsatellite markers: 28 from Population 1, 30 from Population 2, and 31 from Population 3. The total number of alleles ranges from 1 to 23 per locus with on average of 10 alleles per locus (Table 1). One marker (WGU6-5) is monomorphic across all individuals (Table 1). Allelic diversity is high with a total number of 142 alleles across the 14 loci (Table 2). The total number of alleles per population ranges from 74 to 94 with mean number of 86 alleles per population across the species.

Allelic richness ranges from 5.286 (Population 1) to 6.714 (Population 2) with an average of 6.119 across the species (Table 2). Private alleles range from 22.4% (20, Population 3) to 28% (21, population 1) with an average of 24.7% (21).

Individuals with multi-locus genotypes are defined as individuals that share a combination of more than 2 alleles. This can be informative in that it can define what individuals are clones of each other. Multi-locus genotype data can be analyzed using measures of genetic distance, such as Bruvo's distance (Bruvo et al 2014) to determine the degree of differentiation between individuals. Of the eight-nine individuals of *Geum geniculatum* eighty-eight (98.9%) individuals exhibit their own unique multi-locus genotype (Table 2).

Mean observed heterozygosity across the species 0.706 and ranges from 0.675 (Population 1) to 0.763 (Population 2; Table 2). The expected heterozygosity across the species ranges from 0.571 (Population 1) – 0.615 (Population 2) within populations and the mean is 0.591 (Table 2). The inbreeding coefficients test for Hardy-Weinberg equilibrium F_{IS} statistic was calculated from an AMOVA (Excoffier et al. 1992, Michalakis and Excoffier 1996) and 999 Monte Carlo permutations. In *G. geniculatum* it ranges from -0.386 (Population 2) to -0.439 (Population 1; Table 2) and is -0.407 across the species. Heterozygosity-based inbreeding coefficient G_{IS} (Nei 1987) run at 999 Monte Carlo permutations ranges from -0.240 (Population 2) to -0.160 (Population 3) and is -0.194 across the species (Table 2). All values of G_{IS} and F_{IS} were significant ($P = 0.001$).

Table 1. Microsatellite markers used, and number of alleles observed in *Geum geniculatum*.

Locus		Observed Number of Alleles
7389	Hamann et al. 2014	14
11534	Hamann et al. 2014	2
13198	Hamann et al. 2014	23
14769	Hamann et al. 2014	2
WGU1-33	Arens et al. 2004	10
WGU2-10	Arens et al. 2004	17
WGU2-28	Arens et al. 2004	2
WGU2-48	Arens et al. 2004	15
WGU3-15	Arens et al. 2004	18
WGU5-11	Arens et al. 2004	7
WGU5-12	Arens et al. 2004	6
WGU6-1	Arens et al. 2004	7
WGU6-23	Arens et al. 2004	18
WGU6-5	Arens et al. 2004	1
	Mean	10.142
	Total	142

Table 2. Genetic diversity of *Geum geniculatum* across its range.

	N	A	A_e	AR	P	H_o	H_e	F_{IS}	G_{IS}	M
Population 1	28	74	3.215	5.286	18	0.675	0.571	-0.439	-0.182	27
Population 2	30	94	3.782	6.714	25	0.763	0.615	-0.386	-0.240	30
Population 3	31	89	3.450	6.357	20	0.679	0.585	-0.397	-0.160	31
Mean	30	86	3.301	6.119	21	0.706	0.591	-0.407	-0.194	29
Total	89	142								88

N sample size, *A* total number of alleles per population, A_e effective number of allleles, *AR* allelic richness, *P* private alleles per population, H_o observed heterozygosity, H_e expected heterozygosity, and F_{IS} Wright's inbreeding coefficient, G_{IS} Nei's inbreeding coefficient and *M* Number of multilocus genotypes

Genetic structure. Clustering analyses are used to identify structure among populations and is beneficial to visualize gene flow between populations. A series of clustering analyses were performed for *Geum geniculatum*. The first was a discriminant analysis of principal components (Jombart et al. 2010). Discriminant analysis of principal components (DAPC) is a type of multivariate clustering analysis similar to principal components analysis (PCA). PCA ignores known patterns of the individuals such as shared phenotype or origin and plots the data based on

variation within the data. DAPC uses the principals of a PCA but takes it further by assigning individuals to groups using K-means clustering using Bayesian Information Criterion to identify the discrete clusters and providing graphical visualization between population relationships (Jombart et al. 2010). The DAPC (Figure 2) exhibits three distinct clusters. Each cluster is made up of only individuals from the mountains they were sampled. The minimal spanning tree on the DAPC indicates that Populations 1 and 2 are more closely related to Population 3 than each other.

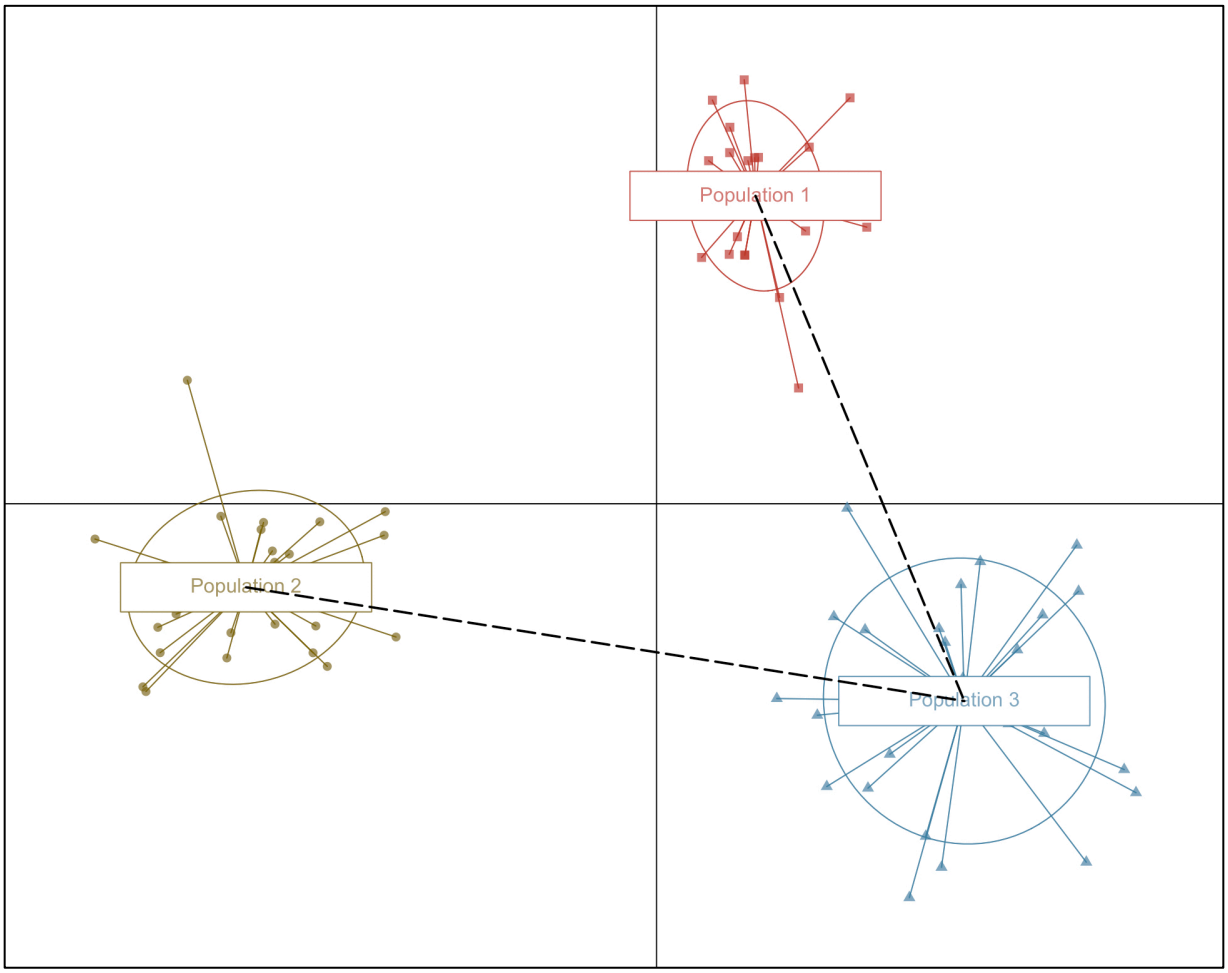

Figure 2. Discriminant Analysis of Principle components using Bruvo's distance. The dashed lines indicates a minimal spanning tree showing relationship between clusters.

Neighbor joining trees are unrooted and have been found to be efficient in identifying clusters and relationships between individuals (Saitou and Nei 1987). The neighbor joining tree built for *G. geniculatum* uses Bruvo's distance to identify genetic distance between individuals. Bruvo's distance is a measure of genetic distance that accounts for the stepwise mutation process of microsatellite loci and has been found to be the most appropriate for polyploid species (Bruvo et al. 2004). The tree contains three distinct branches, each branch consisting of only individuals collected from the same mountain (Figure 3).

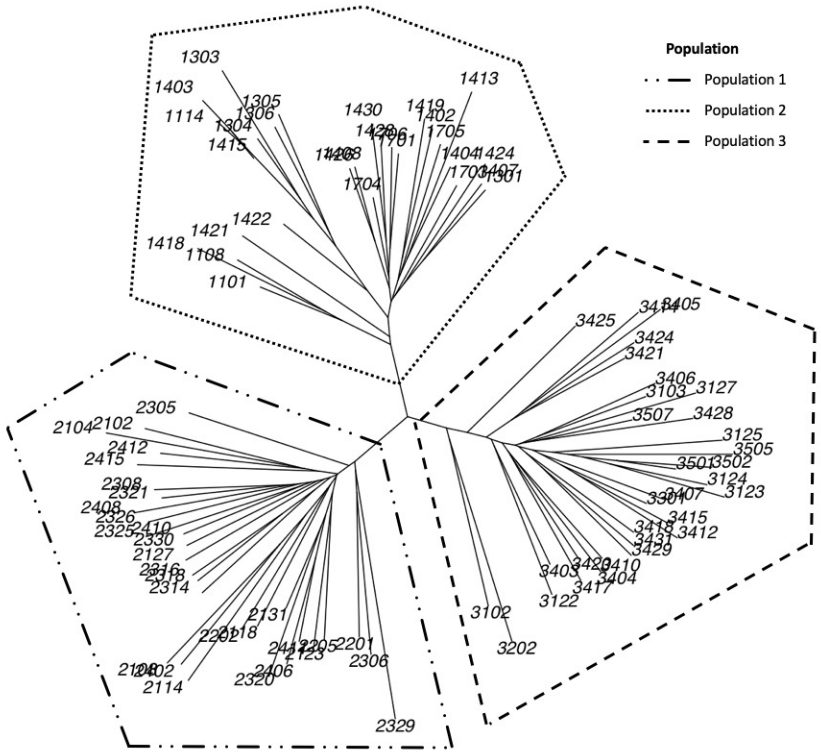

Figure 3. Neighbor joining tree using Bruvo's distance. Individuals from within the same mountain are outlined.

A minimum spanning network of multilocus genotypes based on Bruvo's distance was also constructed (Figure 4), where each node represents a distinct multilocus genotype (MLG = 88) and their relationship on the tree is based on genetic distance (Fig 3). This analysis also

suggests three distinct clusters with individuals from Population 2 being at the top of this network, followed by individuals from population 1 and then Population 3. Interestingly, 10 individuals from the three mountains have formed their own network between Populations 1 and 3: 5 individuals from Population 1, 4 individuals from Population 3 and 1 individual from Population 2. Only two individuals from Population 1 shared a multilocus genotypes.

The STRUCTURE analysis identified three clusters (K=3; Figure 5) that correspond with the geographic locations from which they were collected (Figure 1, Figure 6). Of the individual assignments 10.1% (9 of 89) were lower than 95% (Table 3). The population that displayed the greatest amount of admixture from the other populations was Population 3 but interestingly individual assignment was never lower than 55%.

Population differentiation and reduction in population size. F_{ST} values between 0-0.05 are interpreted with little genetic differentiation, moderate differentiation between 0.05-0.15 and high differentiation between 0.15 and 0.25, greater than 0.25 is interpreted as very high genetic differentiation (Balloux and Lugon-Moulin 2002). F_{ST} values ranged from 0.083 to 0.089 between populations. Interpretations of G'_{ST} and Jost's D are on a uniform scale: 0 indicates the least amount of differentiation while 1 indicates complete differentiation (Alcala and Rosenberg 2019). G'st ranged from 0.131 to 0.132 (Table 4). Jost's D ranged from 0.209 to 0.228 (Table 5).

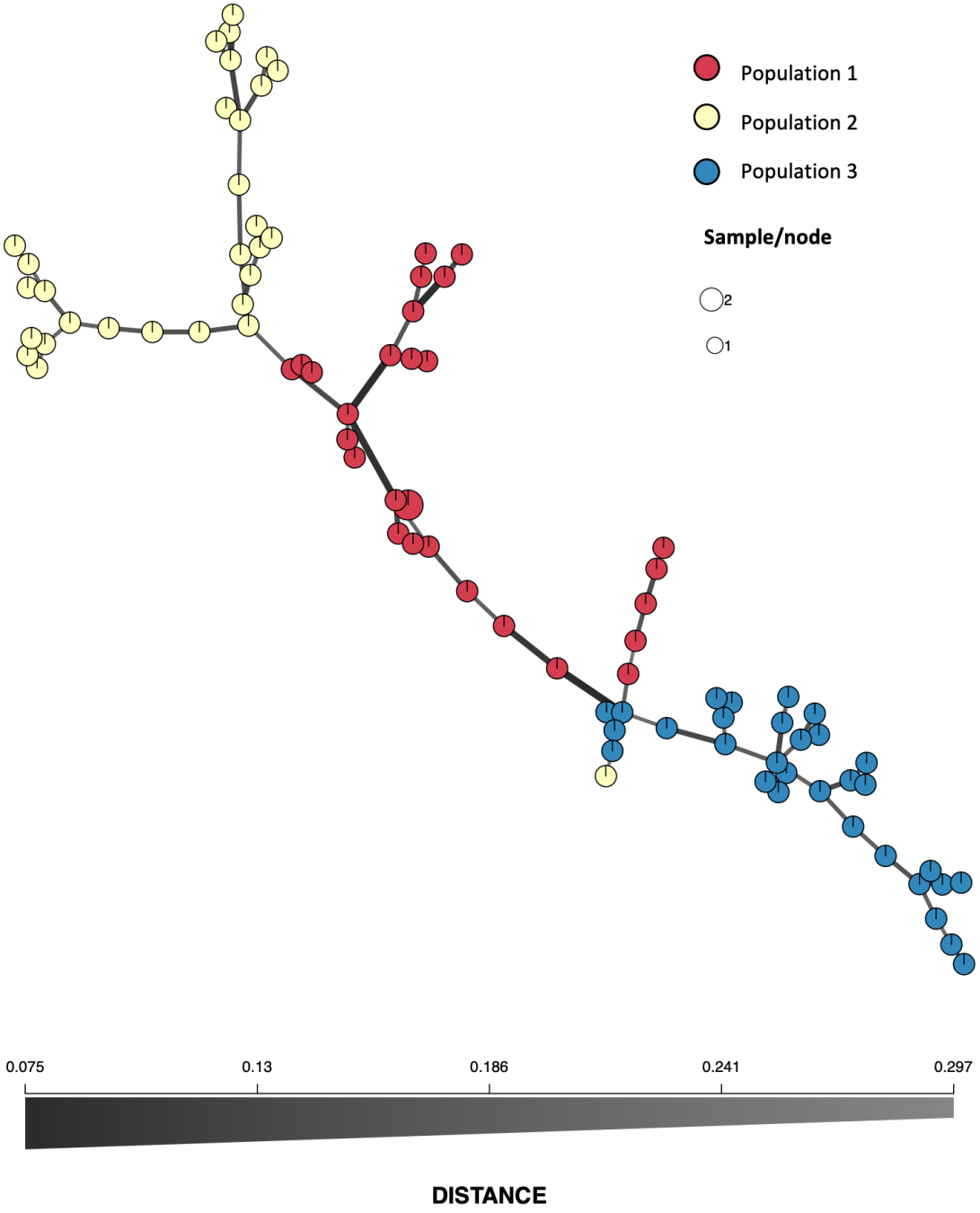

Figure 4. Neighbor joining tree of multilocus genotypes each node indicates a multilocus genotype and the size of the node indicates how many individuals share that genotype. Color of the node indicates what population the individual came from. The thickness and shading of the lines indicate Bruvo's genetic distance between individuals.

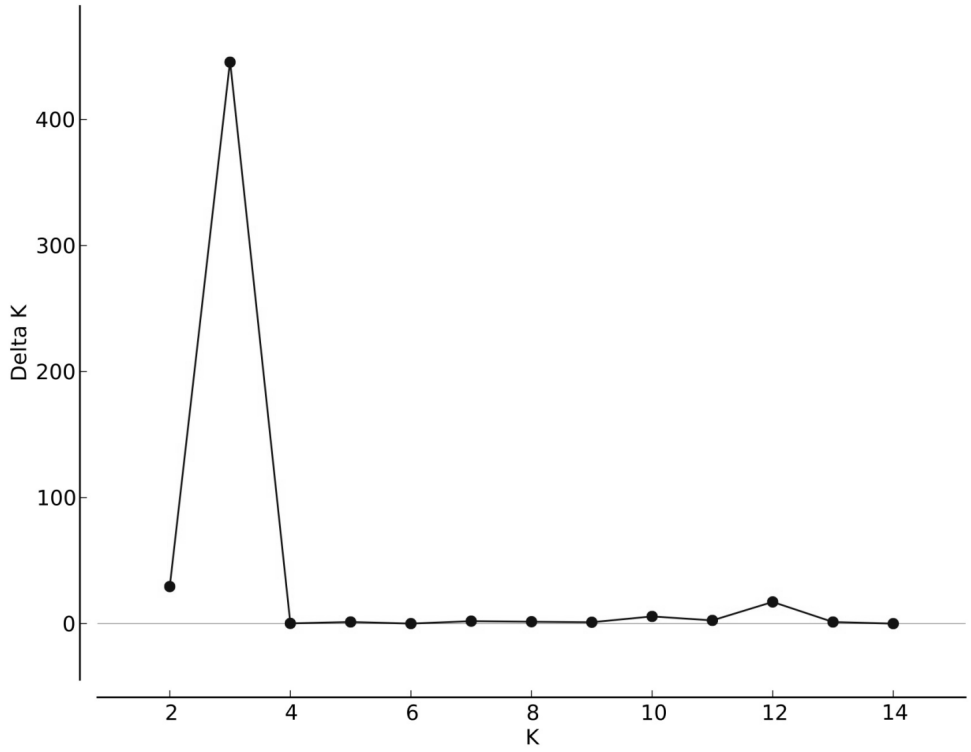

Figure 5. Delta K values calculated using the Evanno Method in Structure Harvester, displaying strongest value of K = 3.

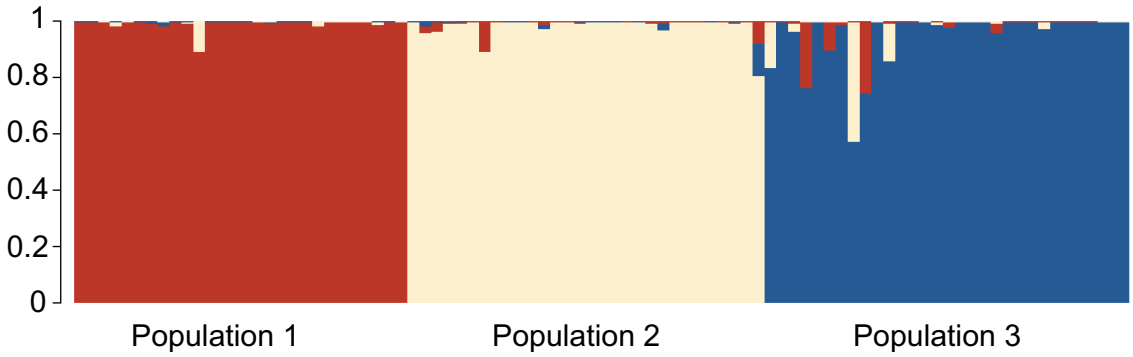

Figure 6. Clustering of individuals using STRUCTURE analysis with a K = 3.

Table 3. Q-matrix of individuals showing less than 95% assignment to their original population.

Individual	Original Population	P1	P2	P3
1407	1	0.892	0.107	0.001
2127	2	0.106	0.893	0.001
2415	2	0.082	0.804	0.114
3102	3	0.005	0.163	0.832
3123	3	0.235	0.002	0.763
3125	3	0.098	0.004	0.897
3202	3	0.004	0.423	0.573
3301	3	0.255	0.001	0.744
3404	3	0.01	0.131	0.859

Table 4. Pairwise table indicating F_{ST} values between populations.

	Population 1	Population 2	Population 3
Population 1	-	0.083	0.089
Population 2	0.083	-	0.089
Population 3	0.089	0.089	-

Table 5. Pairwise table indicating G'_{ST} values between populations.

	Population 1	Population 2	Population 3
Population 1	-	0.131	0.132
Population 2	0.131	-	0.132
Population 3	0.132	0.132	-

Table 6. Pairwise table indicating Jost's D values between populations.

	Population 1	Population 2	Population 3
Population 1	-	0.221	0.209
Population 2	0.221	-	0.228
Population 3	0.209	0.228	-

An M-ratio test was performed to determine if a bottleneck has occurred in populations of *G. geniculatum.* To determine the critical value, theta ($\theta = 0.43$) was approximated using 'strataG' package in R (Archer et al. 2016). Coalescent simulation of a stepwise mutation model was used to estimate the critical M-Ratio value (M_c) taking into account theta, sample size and number of loci. It ran over 10,000 simulations, then takes the 9501 value as critical (Garza and Williamson 2001). If the M-ratio value falls below this number, we can infer the population has undergone a bottleneck. Critical M value (M_c) was 0.847. M-ratio values were very low for all populations (< 0.04) and were less than calculated M_c (0.847). This indicates a bottleneck has occurred in all populations (Table 5).

Table 5. Table showing the M-ratio (Garza and Williamson 2001) across Population 1, Population 2 and Population 3 and the M-critical value for *Geum geniculatum.*

	M ratio
Population 1	0.0353
Population 2	0.0396
Population 3	0.0385
Critical M	0.8474

Discussion

Genetic Diversity. Summary statistics revealed that allelic richness was high in *G. geniculatum* for the species with a total number of 142 alleles across the 14 loci, there was a similar number of alleles (141) found in microsatellite analysis using 8 markers in *G. radiatum,* (Hay et al. 2019). These high numbers of alleles in both species of *Geum* is likely due to it being a hexaploid (2n = 6x = 42) thus having more opportunity for mutations than a diploid organism. Mean allelic richness in *G. geniculatum* was 6.119 and was also similar to *G. radiatum* (6.375) however both were much greater than *G. urbanum* (2.115) occurring in a fragmented landscape (Vandepitte et al. 2007). *Geum radiatum* was overall more diverse than *G. geniculatum* which may be due to there being more populations of *G. radiatum* than *G. geniculatum.*

Polyploids, such as *Geum geniculatum*, are likely to have a higher frequency of alleles than diploids and thus higher numbers of possible genotypes within the same locus (De Silva et al. 2005). Because of this; polyploids typically have increased heterozygosity compared to diploid species (Soltis and Soltis 2000). Gene dosage or the number of copies of each allele at each locus is not known for *G. geniculatum* therefore diversity statistics such as heterozygosity need to be interpreted with caution (Meirmans et al. 2018). In *G. geniculatum,* observed heterozygosity was high across all populations of the species indicating that all populations have high genetic variation. Heterozygosity values vary across different species of *Geum,* expected heterozygosity was high across all populations of *G. radiatum* (Hay et al. 2019) and were also high in *G. geniculatum*'s sister taxa *G. rivale* (Ruhsam et al. 2010). However, *G. urbanum* displayed lower observed heterozygosity than expected heterozygosity (Ruhsam et al. 2010). In a study using RAPD markers, the clonal species *G. urbanum* was also found to have overall low expected heterozygosity (Pluess and Stocklin 2004).

The observed heterozygosity was also higher than the expected heterozygosity. To test if this difference is significant, F_{IS} and G_{IS} were run using 999 Monte Carlo permutations (Meirmans et al. 2018). The results for F_{IS} and G_{IS} were significant and negative indicating there is an excess of heterozygosity across all populations likely due to its polyploid nature. This negative inbreeding coefficient also indicates there is avoidance of consanguinity and suggests that *G. geniculatum*'s breeding system is outcrossing with self-incompatibility (Wright 1965, Balloux 2004).

Another indication of high genetic diversity is that there were 88 unique multi-locus genotypes across 89 individuals. These individuals share a distinct combination of alleles across the 14 loci. From these results we can infer that there is little clonal reproduction in the species which further supports outcrossing as a dominant breeding system for the species. Interestingly, Ruhsam et al. (2010) found that populations of *Geum rivale* in Europe was a predominant outcrosser and possesses a mechanism that results in abortion of flowers when self-pollinated.

The discriminant analysis of principal components (DAPC) uses Bruvo's distance, indicates three genetically isolated populations. Individuals from Population 1 are more tightly clustered together than individuals from Population 2 and Population 3. This indicates that these individuals are more closely related than individuals from the latter populations. Individuals from Population 3 show the greatest distances between each other in comparison to individuals from the other two populations. A minimal spanning tree on the DAPC indicates that Populations 1 and 2 are more closely related to Population 3 than to each other. This provides evidence for historic gene flow between the populations.

The neighbor-joining tree and minimum spanning network of multi-locus genotypes also utilize Bruvo's distance and exhibits three distinct clusters consisting only of individuals from

their population of origin. In the minimum spanning network Populations 2 and 3 are more closely related to Population 1. This result differs from the results of the DAPC indicating Populations 1 and 2 were more distantly related to each other than to Population 3. In the minimum spanning network, there are ten individuals that have formed their own group. These individuals are from all three populations and are closely related to one another. This could be additional evidence indicating these populations were once part of a larger population.

The results of the STRUCTURE analysis (Fig 5, Fig 6) support 3 distinct clusters (K=3) consisting of individuals from their populations of origin. The majority of individuals were assigned with greater than 90% probability back to their populations of origin. However, some individuals from Population 3 exhibited mixed assignments from Populations 1 and 2.

Clustering analyses have the ability to elucidate possible cryptic populations regardless of geographic location (Pritchard et al. 2000). Discriminant analysis of principal components (DAPC), neighbor-joining tree, minimum spanning network and STRUCTURE, all confirm that all three mountains are genetically isolated from one another and individuals are more closely related to individuals from within populations than between populations. These clustering results support the idea that these three populations are acting as distinct metapopulations with little to no geneflow occurring between them. However, although clearly structured and genetically isolated they were once part of a more contiguous population. This supports the hypothesis that *G. geniculatum* is a post-Pleistocene relict species and they likely sought refuge at these high elevations ultimately becoming genetically isolated from one another.

Genetic drift and isolation. Genetic drift along with natural selection and gene flow are all mechanisms that create alterations in allele frequencies within populations and can therefore facilitate speciation (Andrews 2010). Unlike selection, genetic drift is a stochastic process which

leads to a random change in allele frequencies and is not affected by selection (Allendorf et al. 2013). When populations are large or in equilibrium the effects of genetic drift are not readily observed, however, in small, isolated populations, such as that with rare species, drift can cause a profound change in allele frequencies, decrease genetic variation and lead to fixation of alleles (i.e. private alleles; (Star and Spencer 2013). *Geum geniculatum* occurs in a few highly fragmented populations however is found in large population sizes which is likely maintaining the high allele frequencies and high heterozygosity in the species. The high numbers of private alleles strongly suggest that genetic drift is driving differentiation in these populations.

Bottlenecks are forms of genetic drift that can cause severe effects in small or reduced populations. A bottleneck is when a random event occurs wiping out the majority of the population leaving only a small number of individuals remaining, resulting in an extreme loss of heterozygosity (Hartl & Clark 1997; Allendorf et al. 2013). This leads to an immediate loss of allelic variability, particularly in rare alleles but can cause excess of heterozygotes due to a reduction of effective population size (Allendorf et al. 2013). The M-ratio test can be used to identify if a bottleneck has occurred. Garza and Williamson (2001) found that M continues to decrease if populations remain small and isolated after a bottleneck. This is because in small populations the total number of alleles can only increase due to mutation. In the case of *G. geniculatum* the M-ratio test was less than that of the critical value indicating bottleneck has occurred among all populations. While having experienced a bottleneck populations sizes of *G. geniculatum* are abundant which likely attributes the maintenance of high genetic diversity across these populations.

In addition to tests for bottleneck, there are other signs pointing to genetic drift in populations of *G. geniculatum.* First, the populations are heterogeneous on the landscape and all

populations have high numbers of private alleles or alleles that have become fixed within the population. Genetic drift results in fixation of alleles or the accumulation of private alleles within isolated population (Kimura and Ohta 1969, Garza and Williamson 2001). Populations are moderately differentiated (F_{ST}, G'_{ST}, Jost's D) which also indicates there is some level of genetic drift occurring (Balloux and Lugon-Moulin 2002, Alcala and Rosenberg 2019). Moderate differentiation was also observed between populations of *Geum radiatum*, *Carex misera, Trichophorum cespitosum,* and *Calamagrostis cainii* which share the same range as *G. geniculatum* but occur in high elevation rock outcrop communities (Godt et al. 1996).

In the discriminant analysis of principle components, the individuals are clearly more related to other individuals from within populations than to individuals from outside of their population. There is further evidence of this in the STRUCTURE analysis (Figure 2) where K values are identifying 3 distinct clusters corresponding to the individual mountains, only. This isolation has likely been the cause of genetic drift within the species. A similar STRUCTURE pattern was found in *Aquilegia thalictrifolia*, a Pleistocene relict species in Europe, where individuals clustered into the different valleys to which they reside in the Italian Alps (Lega et al. 2014). Lega et al. (2014) attributes this clustering to historic genetic drift over migration and the topography of the landscape impeding on contemporary geneflow. In the southern Appalachians structuring based on geographic isolation has also been seen in *Geum radiatum* where 4 geographically divided clusters roughly based on a North-South-East-West axis (Hay et al. 2019).

Management implications. In the United States, *Geum geniculatum* is a federal species of concern (FSC) but is likely not listed because known populations occur on protected lands

(except for one: EO 32). While there are a number of element occurrences for the species, the data presented here support the conclusion that there are only 3 metapopulations for the species and that genetic drift is driving differentiation. Genetic drift and the loss of genetic variation in small populations has the potential to decrease fitness (Reed & Frankham 2003; Star & Spencer 2013). *Geum radiatum* is known from 14 populations within the same range. It is federally listed and the majority of the populations also occur on protected lands. It is recommended that like *G. radiatum, G. geniculatum* also be federally listed. If federal protection status does not occur, it is imperative for the species that the land in which it resides be preserved. Luckily, Population 1 is owned by the NC Plant Conservation Program and Population 2 occurs on NC State Park land and along the Blue Ridge Parkway. Lastly, most of the land at Population 3 is owned by the USFS: Pisgah National Forest and Cherokee National Forest but is also made up of a number of tracts owned by conservation organizations with the goal of preservation. In agreeance with recommendations by Shawn Oakley in 1991, care needs to be taken if resources such as timber are to be extracted from any of these sites and a buffer along subpopulations in which it resides should be accounted for to minimize erosion of habitat and maintenance of preferred canopy cover (90-100%).

Compared to previous observations, some population sizes seem to have decreased, however it is not known how previous surveys were conducted therefore comparisons need to be made with caution. The abundance of individuals within populations is likely maintaining its high genetic diversity. However, if population sizes continue to decrease augmentation to boost numbers of individuals may be beneficial. If this is to occur however, each population should be treated individually and individuals grown need to be planted from their appropriate evolutionary significant unit i.e. mountain origin. Genetic results indicate that populations of *Geum*

geniculatum are highly structured, with high percentage of private alleles and there is little to no gene flow occurring between them. Because of this structuring populations have likely adapted to their individual mountains and bringing in genetic material from another population could break these adapted genotypes and reduce fitness (Storfer 1999).

Acknowledgements

The author would like to acknowledge USFS, National Park Service, NC State Parks, NC Plant Conservation Program, TN Department of Environment and Conservation, Southern Appalachian Highlands Conservancy and NC Chapter of the Nature Conservancy for issuing permits for collections. Drs. Jennifer Rhode Ward and Ray Williams provided continued support as committee members and mentors. Dr. Chris Ulrey from the Blue Ridge Parkway and Sue Fruchey from the US Forest Service for guidance and help in the field. Page Mangum, Alyssa Phillips and Logan Clark for their assistance and support in the field and in the lab. This project would not be possible if not for funding from the Shinn Scholarship through the NC Native Plant Society, Appalachian State University Office of Student Research and Appalachian State University Student Government Association.

Literature Cited

Alcala, N., and N. A. Rosenberg. 2019. G ST ' , Jost's D, and FST are similarly constrained by allele frequencies: A mathematical, simulation, and empirical study. Mol Ecol **28**:1624-1636.

Allendorf, F. W., G. Luikart, and S. N. Aitkin. 2013. Conservation and the Genetics of Populations. 2nd edition. Wiley-Blackwell

Archer, F., P. Adams, and B. Schneiders. 2016. strataG: An R package for maniuplating, summarizing and analyzing genetic data. Molecular Ecology Resources **17**: 5-11.

Arens, P., W. Durka, J. H. Wernke-Lenting, and M. J. M. Smulders. 2004. Isolation and characterization of microsatellite loci in *Geum urbanum* (Rosaceae) and their transferability within the genus Geum. Molecular Ecology Notes **4**:209-212.

Balloux, F. 2004. Heterozygote Excess in Small Populations and the Heterozygote-Excess Effective Population Size. Evolution **58**:1891-1900.

Balloux, F., and N. Lugon-Moulin. 2002. The estimation of population differentiation with microsatellite markers. Molecular Ecology **11**:155-165.

Bruvo, R., N. K. Michiels, T. G. D'Souza, and H. Schulenburg. 2004. A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. Molecular Ecology **13**:2101-2106.

Clark, L. V., and A. D. Schreier. 2017. Resolving microsatellite genotype ambiguity in populations of allopolyploid and diploidized autopolyploid organisms using negative correlations between allelic variables. Molecular Ecology Resources **17**:1090-1103.

Cogbill, C. V., and P. S. White. 1991. The Latitude-Elevation Relationship for Spruce-Fir Forest and Treeline along the Appalachian Mountain Chain. Vegetatio **94**:153-175.

De Silva, H. N., A. J. Hall, E. Rikkerink, M. A. McNeilage, and L. G. Fraser. 2005. Estimation of allele frequencies in polyploids under certain patterns of inheritance. Heredity (Edinb) **95**:327-334.

Delcourt, H. R., and P. A. Delcourt. 1988. Quaternary landscape ecology: Relevant scales in space and time. Landscape Ecology **2**:23-44.

Delcourt, P. A., and H. R. Delcourt. 1987. Late-quaternary dynamics of temperate forests: applications of paleoecology to issues of global environmental change. Quaternary Science Reviews **6**:129-146.

Delcourt, P. A., and H. R. Delcourt. 1998. Paleoecological Insights on Conservation of Biodiversity: A Focus on Species, Ecosystems,and Landscapes. Ecological Applications, **8**:921-934.

Earl, D. A., and B. M. vonHoldt. 2011. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources **4**:359-361.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology **14**:2611-2620.

Excoffier, L., P. E. Smouse, and J. M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics **131**:479-491.

Gajewski, W. 1957. A Cytogenetic Study on the Genus *Geum* L. Monographiae Botanicae **4**:1-416.

Garza, J. C., and E. G. Williamson. 2001. Detection of reduction in population size using data from microsatellite loci. Molecular Ecology **10**:305-318.
Godt, M. J. W., B. R. Johnson, and J. L. Hamrick. 1996. Genetic Diversity and Population Size in Four Rare Southern Appalachian Plant Species. Conservation Biology **10**:796-805.
Gonzales, E., J. L. Hamrick, and S.-M. Chang. 2008. Identification of glacial refugia in south-eastern North America by phylogeographical analyses of a forest understorey plant, *Trillium cuneatum*. Journal of Biogeography **35**:844-852.
Hale, M. L., T. M. Burg, and T. E. Steeves. 2012. Sampling for microsatellite-based population genetic studies: 25 to 30 individuals per population is enough to accurately estimate allele frequencies. PLoS One **7**:e45170.
Hamann, E., H. Kesselring, J. Stocklin, and G. F. Armbruster. 2014. Novel microsatellite markers for the high-alpine *Geum reptans* (Rosaceae). Appl Plant Sci **2**.
Hay, N.M., Ulrey C., Kauffman G., Murrell Z.E., and Estep M.C. 2019. The genetic diversity and population structure of *Geum radiatum*: effects of past augmentation of an endangered hexaploid. (in press @ Castanea)
Jombart, T. 2008. adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics **24**:1403-1405.
Jombart, T., S. Devillard, and F. Balloux. 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genetics **11**.
Jost, L. 2008. G(ST) and its relatives do not measure differentiation. Mol Ecol **17**:4015-4026.
Kamvar, Z. N., J. C. Brooks, and N. J. Grunwald. 2015. Novel R tools for analysis of genome-wide population genetic data with emphasis on clonality. Front Genet **6**:208.
Kamvar, Z. N., J. F. Tabima, and N. J. Grunwald. 2014. Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. PeerJ **2**:e281.
Kearse, M. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics **28**:1647-1649.
Kimura, M., and T. Ohta. 1969. The Average Number of Generations until Fixation of a Mutant Gene in a Finite Population. Genetics **61**:763-771.
Lega, M., S. Fior, M. Li, S. Leonardi, and C. Varotto. 2014. Genetic Drift Linked to Heterogeneous Landscape and Ecological Specialization Drives Diversification in the Alpine Endemic Columbine *Aquilegia thalictrifolia*. J Hered **105**:542-554.
Meirmans, P. G., S. Liu, and P. H. van Tienderen. 2018. The Analysis of Polyploid Genetic Data. J Hered **109**:283-296.
Meirmans, P. G., and P. H. v. Tienderen. 2004. GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. Molecular Ecology Notes **4**:792-794.
Michalakis, Y., and L. Excoffier. 1996. A Generic Estimation of Population Subdivision Using Distances Between Alleles With Special Reference for Microsatellite Loci. Genetics **142**.
NatureServe. 2018. NatureServe Explorer: An online encyclopedia of life [web application]. NatureServe, Arlington, VA. USA.
NCNHP. 2018. North Carolina Natural Heritage Program Element Occurences. Natural Heritage Program.
Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York, NY.
Oakley, S. 1991. Element Stewardship Abstract for *Geum geniculatum*. The Nature Conservancy, Arlington, VA.

Petrova, A., D. Petrović, D. Soljan, and V. Stevanović. 2011. *Geum bulgaricum*. The IUCN Red List of Threatened Species 2011: e.T165125A5977317. International Union for Conservation of Nature and Natural Resources.
Pluess, A. R., and J. Stocklin. 2004. Population genetic diversity of the clonal plant *Geum reptans* (Rosaceae) in the Swiss Alps. American Journal of Botany **91**:2013-2021.
Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics **155**:945-959.
R Core Team. 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Ramasamy, R. K., S. Ramasamy, B. B. Bindroo, and V. G. Naik. 2014. STRUCTURE PLOT: a program for drawing elegant STRUCTURE bar plots in user friendly interface. Springerplus **3**:431.
Raynor, L. A. 1952. Cytotaxonomic Studies of *Geum*. American Journal of Botany **39**:713-719.
Robinson, L. G., and J. T. Finnegan. 2016. Natural Heritage Program List of Rare Species of North Carolina.*in* N. C. D. o. N. a. C. Resources, editor.
Ruhsam, M., P. M. Hollingsworth, J. Squirrell, and R. A. Ennos. 2010. Significant differences in outcrossing rate, self-incompatibility, and inbreeding depression between two widely hybridizing species of Geum. Biological Journal of the Linnean Society **101**:977-990.
Russell, D. A., F. J. Rich, V. Schneider, and J. Lynch-Stieglitz. 2009. A warm thermal enclave in the Late Pleistocene of the South-eastern United States. Biological Reviews **84**:173-202.
Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution **4**:406-425.
Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol **18**:233-234.
Smedmark, J. E. E., and T. Eriksson. 2002. Phylogenetic Relationships of *Geum* (Rosaceae) and Relatives Inferred from the nrITS and trnL-trnF Regions. American Society of Plant Taxonomists **27**:303-317.
Soltis, P. S., and D. E. Soltis. 2000. The role of genetic and genomic attributes in the success of polyploids. Proceedings of the National Academy of Sciences of the United States of America **97**:7051-7057.
Star, B., and H. G. Spencer. 2013. Effects of genetic drift and gene flow on the selective maintenance of genetic variation. Genetics **194**:235-244.
Storfer, A. 1999. Gene flow and endangered species translocations: a topic revisited. Biological Conservation **87**:173-180.
Taylor, K. 1997. Geum Rivale L. Journal of Ecology **85**:721-731.
Ulrey, C., P. F. Quintana-Ascencio, G. Kauffman, A. B. Smith, and E. S. Menges. 2016. Life at the top: Long-term demography, microclimatic refugia, and responses to climate change for a high-elevation southern Appalachian endemic plant. Biological Conservation **200**:80-92.
Vandepitte, K., H. Jacquemyn, I. Roldan-Ruiz, and O. Honnay. 2007. Landscape genetics of the self-compatible forest herb Geum urbanum: effects of habitat age, fragmentation and local environment. Mol Ecol **16**:4171-4179.
Vieira, M. L., L. Santini, A. L. Diniz, and F. Munhoz Cde. 2016. Microsatellite markers: what they mean and why they are so useful. Genet Mol Biol **39**:312-328.
Walker, M. J., A. K. Stockman, P. E. Marek, and J. E. Bond. 2009. Pleistocene glacial refugia across the Appalachian Mountains and coastal plain in the millipede genus *Narceus*:

evidence from population genetic, phylogeographic, and paleoclimatic data. BMC Evol Biol **9**:25.
Weakley, A. S. 2015. Flora of the Southern and Mid-Atlantic States. University of North Carolina Herbarium, North Carolina Botanical Garden, University of North Carolina, Chapel Hill.
Wiser, S. K. 1994. High-Elevation Cliffs and Outcrops of the Southern Appalachians: Vascular Plants and Biogeography. Castanea **59**:85-116.
Wright, S. 1965. The Interpretation of Population Structure by F-Statistics with Special Regard to Systems of Mating. Evolution **19**:385-420.

Chapter 5: Discussion

Census and Demography

Geum geniculatum is a perennial herb endemic to the high elevations (> 1200 m) of three mountains between North Carolina and Tennessee. Its heterogeneous distribution within this area is likely due to historic climactic changes of warming and drying periods during the Pleistocene where the moist peaks of high elevations acted as refuge for the species (Weakley, 2015). It also may provide novel ecosystem services within these high elevations that, if lost, would alter interspecific dynamics on these high elevation habitats.

During the summer of 2018, 13 of the known element occurrences were visited. Its observed growth habit is in clumps dispersed throughout the landscape, typically along stream banks, within boulderfield forests, along trails and grassy balds. In one location, it is growing alongside *Geum canadense*, a more common species and at another the species abruptly stops growing and is replaced with *G. canadense*. In agreeance with previous findings the most robust populations were found on streambanks in northern hardwood and rich cove forests with ~5-10% sunlight (Ch 2 – Table 2; Oakley, 1991). However, population sizes observed were less than previously reported to the NC Natural Heritage Program. This could be due to different censusing techniques where previous observations were based on estimates of population size and the methods used in this study were counts of individuals. It is still important to consider this because reduction in population sizes of *G. geniculatum* will ultimately lead to a loss of genetic variability and could also reduce the species ability to adapt to the pressures of our changing climate.

The demography study established in July 2019 is not fully representative of life history traits of the entire species due to it being from only one of the mountains; also, the locations at

which it will be monitored are only representative of 2 of the type of habitats it occupies: trailside and grassy bald. Despite this, it will be a good baseline to begin to understand the species and its population viability traits. It is recommended that a level of quantitative monitoring be established at the other two populations to understand stability of the species. This data would also be useful to understand life history across the entire species.

Time-lapse Insect Survey

In all plants, but especially rare plants such as *Geum geniculatum* gene flow via pollen transfer between populations is crucial for long-term viability (Ellstrand, 1992). Gene flow also ensures maintenance of genetic diversity and pollen vectors attribute to genetic structure among plant populations. Plants have evolved a variety of ways in which to transfer pollen, known as breeding systems, these include outcrossing, self-pollination and mixed-mating systems. For those plants that outcross, this typically occurs via pollination. Interestingly, 87.5% of all flowering plants worldwide rely on animal-pollination and the dominant pollinators are insect (Ollerton, Winfree, & Tarrant, 2011). Therefore, it is important to understand breeding systems and pollinators particularly in rare species where populations are at the highest risk to the effects of inbreeding and genetic drift due to highly fragmented population sizes. To begin to learn about the pollination biology of *Geum geniculatum* a time-lapse insect visitor was performed. The hopes of this study is that it will be built upon to understand the full reproductive biology of the species.

In the time-lapse study conducted between the summers of 2018 and 2019, a Brinno 200 HD was set up across the range of *Geum geniculatum.* Videos were reviewed and insect visitation was identified as any insect landing on the inflorescence (Edwards, Smith, & McEntee,

2015). Time spent within the frame, number of flowers visited and number of flowers in bud were also recorded. Insects were identified to the lowest order of classification possible. In the first year of the time-lapse insect survey there were no visitors observed. While flowering time for the species has been documented from late-June to August, the inability to observe visitors is attributed to many of the flowers already forming seed. In the second year the camera was set up earlier in the season and an abundance of visitors were observed. The dominant visitor across the two sites it recorded were *Bombus* sp. At the site that occurred on a grassy bald, *Bombus* sp. was the only visitor observed but a total of 142 visitations were observed across 327 flowers (Ch 3 – Table 2). The second location the camera recorded was along a streambank and three types of insects were observed: *Bombus* sp., Syrphid fly and an unknown species. Overall there were less visitations (23, Ch 3- Table 2) at the second location compared to the first.

Population Genetics

Results of a population genetics study using 14 microsatellite loci reveal an overall diverse species with high genetic diversity, moderate differentiation, and distinct structuring of populations based on mountain. There is evidence that these populations have undergone a population bottleneck based on the M ratio test and the high numbers of private alleles. This structuring across the landscape provides evidence that each mountain is indeed a metapopulation. These metapopulations are acting as evolutionary significant units and therefore should be managed independently (Casacci, Barbero, & Balletto, 2013). Gene flow is likely occurring within these metapopulations through pollen flow or a seed vector but is not often occurring among them. This is likely due to the distances between these populations: As the crow flies Population 1 and Population 2 are separated by ~23 km, Population 2 and Population

3 by ~27 km and Population 1 and Population 3 by ~41.5 km (Google Earth 2019). This distance is likely too great for an insect pollinator to travel, for instance the longest distance *Bombus* sp. has the ability to travel for food is 10 km however prefers to stay as close to the nest as possible (Goulson, 2010).

I suspect the grassy balds at the tops of Populations 1 and 3 are acting as a highway for gene flow between the sides of the mountains. Because of the history of grazing on these balds herbivores likely brought seeds from one drainage to another through the adhesion of seeds to their fur, creating a network of gene flow. In modern times the seed vector is likely humans as the plant is found along heavily used hiking trails.

While the beginning stages of pollinator identification for *G. geniculatum* have begun population genetic data reveals clues into its reproductive strategies. For instance, the species exhibits distinct clustering in DAPC, NJ trees, and STRUCTURE analysis (Chapter 4 – Fig 2, 3, 4, 5) with moderate differentiation (Chapter 4 - Tables 4, 5, 6) based on the mountains from which they occur. This is likely due to historic processes during the Pleistocene that fragmented these populations on these separate mountains. However, genetic diversity statistics reveal high genetic variation within populations and 98% of the individuals sampled revealed a unique multi-locus genotype. There is no evidence of inbreeding within populations of *G. geniculatum* (Chapter 4 – Table 2). These clues indicate a species that is a primary outcrosser whose pollinator has a restriction to gene flow between mountains via topographical barriers.

Further considerations

In the United States, *Geum geniculatum* is a federal species of concern (FSC) but is not listed because most known populations occur on protected lands except for one. While there are

a number of element occurrences for the species, genetic results indicate that there are only 3 metapopulations for the species. *Geum radiatum* (14 populations), its charismatic cousin occurring on high elevation rock outcrops within the same range is federally listed and only known from 14 populations many of which also occur on protected lands. It is recommended that like *G. radiatum, G. geniculatum* also be federally listed. If federal protection status does not occur, it is imperative for the species that the land in which it resides be preserved. Luckily, Population 1 is owned by the NC Plant Conservation Program and Population 2 occurs on NC State Park land and along the Blue Ridge Parkway. Lastly, most of the land at Population 3 is owned by the USFS: Pisgah National Forest and Cherokee National Forest but is also made up of a number of tracts owned by conservation organizations with the goal of preservation. In agreeance with recommendations by Shawn Oakley in 1991, care needs to be taken if resources such as timber are to be extracted from any of these sites and a buffer along subpopulations in which it resides should be accounted for to minimize erosion of habitat and maintenance of preferred canopy cover (90-100%). In addition, protection of any upstream headwaters to prevent erosion or sediment as this has the potential to devastate some of the healthiest populations of *G. geniculatum*.

An effort was made to investigate all of the drainages and streams at Population 1 to find more subpopulations; however, no populations were found at these preferred habitats. Most individuals were found along an old road growing alongside *Geum canadense*. New subpopulations were found on the grassy bald and along an illegal trail that leads to a housing development. Overall, population size was significantly smaller than those found at populations 2 and 3 therefore care needs to be taken to monitor this species over time at Population 1.

There are a number of populations that would benefit from active management. One subpopulation (EO 5) at Population 2 occurs below a campsite which happens to be one of the highest elevations known for the species. This site is being overgrown with *Rubus* sp. which is having a negative effect on the subpopulation there, therefore, it is recommended the *Rubus* sp. be cut back in order to provide more suitable habitat.

Care should be taken when managing the Appalachian Trail at Population 3, *G. geniculatum* has become an opportunist (EO 1) on these trails however has put itself in danger of trampling. There is no appropriate habitat past the trailside therefore if these parts of the trail are to be widened, the entire population could be devastated. Therefore, trail maintenance should be monitored if it is to occur through these sites. The grassy balds are also regularly mowed and at one occurrence on one of the grassy balds there were no plants found, at another only a few individuals were found. Despite this, Population 3 also holds one of the most robust subpopulations (EO 7) observed which is seemingly stable. This subpopulation is easily accessible as the start of the population occurs close to the road. It is recommended that this subpopulation continue to be monitored for population size as it could provide insight into those that are not as easily accessible.

Literature Cited

Casacci, L. P., Barbero, F., & Balletto, E. (2013). The "Evolutionarily Significant Unit" concept and its applicability in biological conservation. *Italian Journal of Zoology, 81*(2), 182-193. doi:10.1080/11250003.2013.870240

Edwards, J., Smith, G. P., & McEntee, M. H. F. (2015). Long-term time-lapse video provides near complete records of floral visitation. *Journal of Pollination Ecology, 16*(13), 91-100.

Ellstrand, N. C. (1992). Gene flow by pollen: implications for plant conservation genetics. *Oikos, 63*(1), 77-86.

Goulson, D. (2010). *Bumblebees : Behaviour, Ecology, and Conservation*. New York NY: Oxford University Press.

Oakley, S. (1991). *Element Stewardship Abstract for Geum geniculatum*. The Nature Conservancy. Arlington, VA.

Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos, 120*(3), 321-326. doi:10.1111/j.1600-0706.2010.18644.x

Weakley, A. S. (2015). *Flora of the Southern and Mid-Atlantic States*: University of North Carolina Herbarium, North Carolina Botanical Garden, University of North Carolina, Chapel Hill.

Vita

Marietta Day Shattelroe was born in Springfield, MA to Alfred Shattelroe and Linda Riordan. She graduated from Northampton High School in Massachusetts in 2006. In 2013, she completed an Associate of Science at Asheville-Buncombe Technical Community College in North Carolina. Following she attended the University of North Carolina at Asheville and was awarded a Bachelor of Science with a concentration in Ecology and Evolutionary Biology in 2015. She began to pursue her Master of Science degree in Ecology and Evolutionary biology in the Fall of 2017. After receiving her M.S. degree in the Fall 2019 she plans to continue to pursue a career in conservation biology and land management.